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Impact of the innate immune response on mammary epithelia

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Impact of the innate immune response on mammary epithelia

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ABSTRACT

Lipocalin 2 (Lcn2) is a member of the lipocalin family, members of which are usually small extracellular proteins with the abilities of binding and transporting small hydrophobic molecules. Lcn2 was named neutrophil gelatinase-associated lipocalin (NGAL) in human, neu related lipocalin (NRL) in rat and SIP24, 24p3, uterocalin, and siderocalin in mouse. The expression level of Lcn2 in the mammary gland is very high during involution. Results in this thesis show that the expression of rat Lcn2 (NRL) is higher in primiparous rat mammary glands after 28 days of involution than its expression in age matched virgin (AMV) mammary glands. Parity is associated with a lower incidence of breast cancer. The population size of macrophages in primiparous mammary glands is also higher in parous glands. More immune surveillance provided by the larger number of macrophages in the mammary gland after involution may be one reason for the observed parity induced protection against breast cancer.

The expression levels of Lcn2 and other inflammatory genes are induced in HC11 cells, a mammary gland epithelial cell line, by mycoplasma infection or the mycoplasma membrane lipopeptide macrophage-activating lipopeptide-2 (MALP-2). Using the Lcn2 promoter linked to a luciferase reporter plasmid, we investigated the mechanism by which MALP-2 regulates gene expression and demonstrated that it activates NFκB and C/EBP and induces IκBζ. Reduction in IκBζ by RNAi reduced Lcn2 promoter activation by MALP-2.

CHAPTER 1. GENERAL INTRODUCTION

1.1 Dissertation Organization

Chapter 1 is a general introduction covering the topics presented in this dissertation. A detailed review of Lcn2 is given first. Following is a review of human breast development and the relationship of pregnancy and breast cancer. A discussion of macrophages and mammary gland development is also included. Next, a review of mycoplasma and its membrane protein macrophage-activating lipopeptide-2 (MALP-2) is presented. The last part of the general introduction is a review of some aspects of inflammation. Chapter 2 is based on my finding of higher expression levels of Lcn2, and several other genes, in fully involuted mammary glands compared with age-matched virgin glands. The results also show that the population size of macrophages is higher in fully involuted mammary glands. In chapter 3, the results of the effect of mycoplasma and MALP-2 on mammary epithelial (HC11) cells are reported. In this section I show that Lcn2 expression by HC11 cells is induced by mycoplasma infection or by the mycoplasma membrane lipopeptide macrophage-activating lipopeptide-2 (MALP-2). MALP-2 is shown to regulate Lcn2 expression by activating NFκB and C/EBP and inducing IκBζ. A general discussion is given in Chapter 4. In the appendix, the work to develop a Lcn2 overexpressing mouse line is described. Thanks to people who have contributed to this thesis are given in the acknowledgment.

1.2 Specific Aims and Significance

Demonstration of a larger Macrophage Population Size and Up-Regulation of Lcn2 in The Fully Involuted Mammary Gland of Rat.

Parity is a demonstrated strong protective factor for breast cancer. However the mechanism of parity induced protection against breast cancer is not clear and the purpose of this study was to investigate its mechanistic basis. The study started with the hypothesis that inflammation was suppressed in the parous breast. The initial analysis of the expression levels of genes that reflect the inflammatory condition of the tissue identified many genes

associated with macrophages that were elevated in primiparous breast tissue compared with mammary tissue from age-matched virgns. This initiated a study to determine whether there are more macrophages in the fully involuted rat mammary gland. Such a finding would be reasonable because increased immune surveillance in the mammary gland by macrophages is a possible reason for protection against breast cancer. Previous studies showed that the number of macrophages in the mouse mammary gland increases during early involution (Atabai et al., 2005; Lund et al., 1996; Stein et al., 2004). The results of a previously published microarray study suggested that there are more macrophages in the mouse mammary gland after the mammary gland has completed its involution compared with age-matched virgin glands (D'Cruz et al., 2002). However, there was no evidence to demonstrate the basis of this difference. The results from this study, in which the macrophage population was assessed by real-time RT-PCR and immunochemical staining, showed that the number of macrophages is higher in fully involuted mammary glands of rats compared with in age-matched virgins. The results also demonstrate that the level of Lcn2 expression is higher in mammary glands after involution. Lcn2 is expressed by epithelial and hematopoietic cell types and has been shown to induce apoptosis in neutrophils but not in macrophages. The persistently high expression of Lcn2 in involuted mammary glands may be one reason that more macrophages but not neutrophils remain in the gland after involution is complete. In summary, I found that the number of macrophages is higher after complete involution of the primiparous rat mammary gland compared to the virgin gland. I propose that the resulting increased immune surveillance in the mammary gland may play a role in protecting the parous gland against breast cancer.

The Effect of Mycoplasma Infection or MALP-2 on HC11 Cells

Mycoplasma infection causes inflammation of epithelial tissues *in vivo* and is a frequent problem in cell cultures. It is important to understand how mycoplasma infection might influence epithelial cell signaling and gene expression. The expression levels of three genes encoding proteins secreted during the innate immune response (Lcn2, IL-6 and TNFα) were found elevated in mycoplasma-infected mammary epithelial (HC11) cells. Expression of these genes was also increased in response to MALP-2, a mycoplasmal membrane

lipopeptide. MALP-2 activates NFκB and C/EBP and induces IκBζ to activate the Lcn2 promoter. Reduction of IκBζ by RNAi reduced Lcn2 promoter activation by MALP-2. Thus, mycoplasma contamination can significantly change the gene expression pattern of epithelial cells, creating a condition that resembles their activation during the innate immune response.

1.3 The Lipocalin Family

Pervaiz and Brew first proposed the grouping and name of the lipocalin family. They used the name "lipocalin" to describe and emphasize the common ability of this group proteins to bind lipophilic molecules. As more members were identified, the lipocalin family was found to be large and diverse (Pervaiz and Brew, 1985; Pervaiz and Brew, 1987). Lipocalins are found in various species including vertebrate and invertebrate animals, plants, insects and even bacteria. Lipocalin family members are usually small (160-180 amino acids) extracellular proteins. The overall amino acid sequence similarity of members may be low, sometimes even lower than the nominal threshold of 20%. However, lipocalins share short conserved sequence motifs. Flower identified three such motifs (also called structurally conserved regions, SCRs) among lipocalins. Most lipocalins, which are also called kernel lipocalins, such as retinol binding protein, β-lactoglobin, and lipocalin 2, share all three SCRs. Other lipocalins, such as the odorant-binding protein, share only one or two SCRs and they are called outlier lipocalins, (Flower et al., 1993; Flower et al., 2000). Despite the low amino acid sequence conservation, lipocalins share a common tertiary structure, which is a symmetric hydrogen bonded β-barrel formed by a single eight stranded anti-parallel β-sheet. The cup-shaped interior, which forms a ligand-binding site, is usually lined with hydrophobic amino acid residues. It has an elliptical shape in cross-section (Flower et al., 2000; Kjeldsen et al., 1996) [Fig. 1].

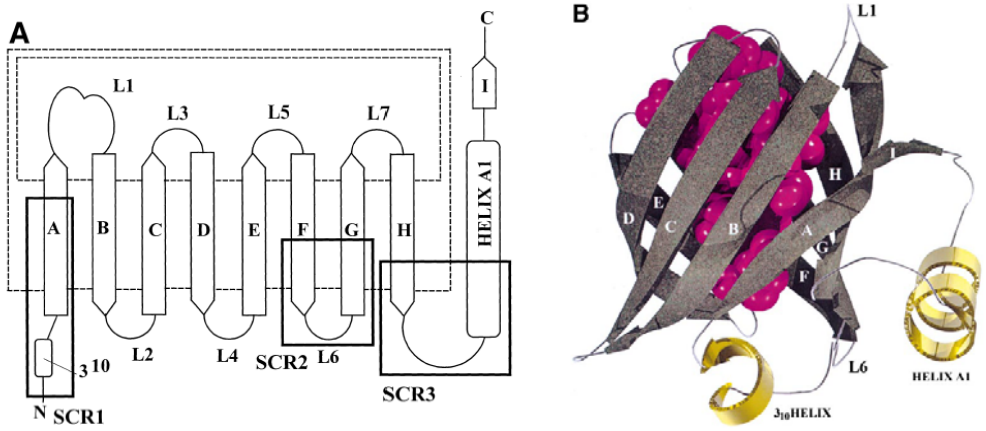

Fig. 1

Figure 1. Structure of the lipocalin fold. (Reprinted from (Flower et al., 2000), Copyright (2000), with permission from Elsevier and with legend taken from the same article)

A) An unwound view of the lipocalin fold orthogonal to the axis of the barrel. The nine β-strands of the antiparallel β-sheet are shown as arrows and labelled A-I. The C-terminal α-helix A1 and N-terminal 310 like helix are also marked. Connecting loops are shown as solid lines and labelled L1-L7. A pair of dotted lines indicates the hydrogen-bonded connection of two strands. One end of the lipocalin L-barrel has four loops (L1, L3, L5, and L7); the opening of the internal ligand-binding site is here and so is called the Open end of the molecule. The other end has three L-hairpin loops (L2, L4 and L6); the N-terminal polypeptide chain crosses this end of the barrel to enter strand A via a conserved 310 helix closing this end of the barrel: the Closed end of the molecule. Those parts that form the three main structurally, and sequence, conserved regions (SCRs) of the fold (SCRl, SCR2, and SCR3) are marked as heavy boxes. SCR3 corresponds closely with the sequence conserved region rather than the structurally conserved region.

B) A schematic or ribbon drawing of the lipocalin fold. The structure shown is a prototypical, rather than an actual, structure. The nine conserved β-strands, the closed β-barrel, and the N-terminal and C-terminal helices are shown (see A above). Loops L1 and L6 are also marked. The size of the ligand binding ultrasite is represented by a collection of atoms, in a purple space-filling depiction, representing the structural overlay of a variety of lipocalin ligands which include retinol, retinoic acid, haem, and bilin. β-Strands are shown as smoothly curving arrows textured as grey granite, α-helices as golden spiral ribbons, and loops as silver cords. Figure generated using ALTER and POVRAY

The common cup-shape structure of lipocalins, which forms a putative binding site composed of both an internal cavity and an external scaffold which is the β-barrel, gives one an impression that they might have the ability to bind something. As a matter of fact, one common property shared by lipocalin proteins is their molecular binding properties. Lipocalins can bind small, usually hydrophobic molecules in the binding site. One example would be plasma retinol binding protein that can bind all-*trans*-retinol, retinal, or retinoic acid (Kanai et al., 1968; Newcomer and Ong, 2000; Ronne et al., 1983). Lipocalins may form covalent or non-covalent complexes with other macromolecules. For example, human neutrophil gelatinase-associated lipocalin (NGAL), the human lipocalin 2, links with neutrophil gelatinase *via* a covalent bond between these two molecules (Kjeldsen et al., 1993). Another capability of lipocalins is that they can bind specific cell surface receptors. Sometimes, one lipocalin protein binds a specific receptor, for example, retinol binding protein binds its specific cell surface receptor and lipocalin 1 binds LIMR (Lcn1 interacting membrane receptor). Sometimes several lipocalins can bind the same receptor, for example, retinol binding protein (RBP), α-1-microglobulin (A1M), mouse major urinary protein (MUP), odorant-binding proteins (OBP) and NGAL can bind megalin, a type I transmembrane protein (Flower, 2000; Hvidberg et al., 2005; Leheste et al., 1999; Wojnar et al., 2001).

Although lipocalins were once classified as transport proteins because of their abilities of binding small hydrophobic molecules and cell surface receptors, more functions of lipocalins are recognized beyond transport. Members of the lipocalin family only share a limited similarity at the amino acid sequence level and have diverse functions, such as cryptic coloration (lepidopteron and crustacean lipocalins), modulation of cell growth and metabolism (apolipoprotein D, α1-microglobulin), regulation of the immune response (α1-microglobulin, glycodelin), smell reception (odorant-binding protein) and animal behaviors (α2-urinary globulin, major urinary protein) (Akerstrom et al., 2000; Flower, 1996; Flower et al., 2000; Kjeldsen et al., 2000).

Lipocalins have also been used as biomarkers for some diseases such as inflammatory diseases, cancer, and lipid disorders. They were demonstrated to have potential clinical applications (Xu and Venge, 2000). For example, prostaglandin D synthase, which is the

only lipocalin that has enzyme activity, has been identified to be a potential marker for brain tumors, renal insufficiency and seminal plasma fertility (Diamandis et al., 1999; Peitsch and Boguski, 1991). Another example is that the α1-microglobulin has been used as a marker for renal tubular injury (Weber and Verwiebe, 1992).

1.4 Lipocalin 2

Lipocalin 2 is member of the lipocalin family and has been identified in human (neutrophil gelatinase-associated lipocalin: NGAL or human neutrophil lipocalin: HNL), rat (neu related lipocalin, NRL), mouse (superinducible protein 24KDa: SIP24, 24p3, uterocalin,, or siderocalin) and chicken (Extracellular Fatty Acid Binding Protein: EX-FABP).

1.4.1 Name, Sequence and Structure

Mouse lipocalin 2 has 200 amino acid residues including the signal peptide (180 without the signal peptide). Mouse Lcn2 was first described as a growth factor induced and superinducible protein (SIP24) produced by mouse 3T3 cells (Nilsen-Hamilton et al., 1982). It was discovered again as an induced gene (24p3) in mouse kidney cells infected by SV40 or polyoma virus (Hraba-Renevey et al., 1989). Flower *et al.* did the protein sequence analysis and they identified the 24p3 protein as a member of lipocalin family by searching conserved sequence motifs (Flower et al., 1991). During pregnancy Lcn2 expression in the mouse uterus increases massively immediately before and peaks immediately after delivery (Liu et al., 1997). In fact, its expression in the uterus around parturition is even higher than its expression in the liver during the acute phase response and that is why it was also named as uterocalin (Liu et al., 1997). The human Lcn2, which was named neutrophil gelatinase-associated lipocalin (NGAL), has 198 amino acid residues with the signal peptide (178 without the signal peptide). It was co-purified with neutrophil gelatinase because a small fraction of NGAL formed a covalent complex with gelatinase. However, most NGAL exists as a monomer or a homodimer and colocalizes with lactoferrin in the neutrophil granules (Kjeldsen et al., 1994; Kjeldsen et al., 1993). The protein was purified as a dimer independently by another group and named as human neutrophil lipocalin (HNL). They found that the protein is associated with secondary granules in neutrophil and the cells

released about 50% of the total cellular NGAL content when exposed to serum-opsonized particles (Xu et al., 1994). Among the sequenced Lcn2 orthologs, NGAL is the only one that forms a covalent bond with itself or other molecules. The reason is that Lcn2 from mouse and rat have only two cysteine residues that form a disulfide bond within the molecule, while NGAL has three cysteine residues that leave a free cysteine residue after forming the disulfide bond. The rat version of Lcn2, which has 198 amino acid with the signal peptide (178 without the signal peptide), was named neu related lipocalin (NRL) due to the 12 fold overexpression of the protein in neu (HER2/c-erbB-2) induced breast cancers but not in ras or chemically induced carcinomas (Stoesz and Gould, 1995). Lcn2 undergoes post translational modification, for example, N-glycosylation was found in the human and mouse Lcn2 proteins (Davis et al., 1991b; Kjeldsen et al., 1993).

Chicken Lcn2 was first described as a 20K transformation sensitive protein secreted by quiescent chicken embryo fibroblasts (Bedard et al., 1989). It was not identified as a lipocalin at that time. Later, another group proposed the name of Extracellular Fatty Acid Binding Protein (EX-FABP), because it binds long-chain unsaturated fatty acids (Cancedda et al., 1996). EX-FABP is a monomer and has a potential glycosylation site, but no glycosylation was found (Descalzi Cancedda et al., 2000).

In 2000, Goetz *et al.* published the NGAL crystal structure. They had three independent NGAL structures: one from dimeric NGAL and two from monomeric NGAL. Although the pH and salt concentration they used for crystallization of the three structures was different, the three NGAL structures were essentially identical. Their results confirmed that NGAL shares the typical lipocalin conserved eight-stranded β-barrel fold, a short 3_{10} helix (residues 24-28) and a four-turn α-helix (residues 145-160). However, the NGAL calyx is larger, wider and more open when compared with other lipocalin structures. Another feature of the NGAL calyx is that it is lined with more polar and positively charged residues than typical lipocalins [Fig 2]. Both the dimeric structure and monomeric structure contain a disulfide linkage between cysteines 76 and 175. In the dimeric structure, there is a disulfide bond between cysteine 87 on each monomer to form the dimer. This disulfide bond is unique for NGAL and not seen in uterocalin or NRL. However, the presence of this disulfide bond does not affect the NGAL crystal structure (Goetz et al., 2000). There is no published crystal

structure for the mouse or rat versions of Lcn2. But it is reasonable to conclude that uterocalin and NRL have very similar structures to NGAL because Lcn2 proteins from different species share a very high sequence homology. For example, NGAL and uterocalin are 62% identical at the amino acid level. NGAL and NRL are 64% identical, uterocalin and NRL are 80% identical [Fig 3.]. In this introduction the following terms will be used to refer to the Lcn2s of different species: uterocalin for mouse Lcn2, NGAL for human Lcn2, NRL for rat Lcn2, and EX-FABP for chicken Lcn2.

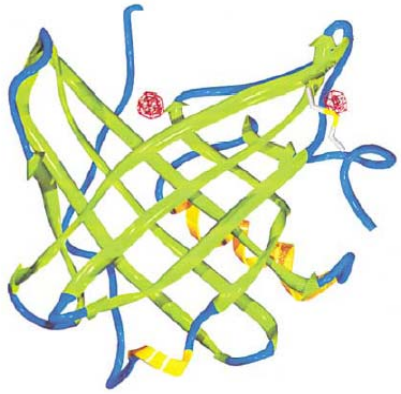

Fig. 2

Figure 2. NGAL Crystal Structure. (Reprinted from (Goetz et al., 2002), Copyright (2002), with permission from Elsevier and with legend taken from the same article)
The 3.4 Å native anomalous difference electron density map is shown contoured at 3.0 σ (red) and 5.5 σ (dark red) superimposed on a ribbon representation of the NGAL structure and colored by secondary structure (helices, yellow; strands, green; coil, blue).

```
50
utc   MALSVMCLGLALLGVLQSQAQDSTQNLIPAPSLLTVPLQPDFRSDQFRGR
NRL   MGLGVLCLALVLLGVLQRQAQDSTQNLIPAPPLISVPLQPGFWTERFQGR
NGAL  MPLGLLWLGLALLGALHAQAQDSTSDLIPAPPLSKVPLQQNFQDNQFQGK
100
utc   WYVVGLAGNAVQKKTEGSFTMYSTIYELQENNSYNVTSILVRDQDQGCRY
NRL   WFVVGLAANAVQKERQSRFTMYSTIYELQEDNSYNVTSILVRGQ--GCRY
NGAL  WYVVGLAGNAILREDKDPQKMYATIYELKEDKSYNVTSVLFRKK--KCDY
150
utc   WIRTFVPSSRAGQFTLGNMHRYPQVQSYNVQVATTDYNQFAMVFFRKTSE
NRL   WIRTFVPSSRPGQFTLGNIHSYPQIQSYDVQVADTDYDQFAMVFFQKTSE
NGAL  WIRTFVPGCQPGEFTLGNIKSYPGLTSYLVRVVSTNYNQHAMVFFKKVSQ
200
utc   NKQYFKITLYGRTKELSPELKERFTRFAKSLGLKDDNIIFSVPTDQCIDN
NRL   NKQYFKVTLYGRTKGLSDELKERFVSFAKSLGLKDNNIVFSVPTDQCIDN
NGAL  NREYFKITLYGRTKELTSELKENFIRFSKSLGLPENHIVFPVPIDQCIDG
```

Fig. 3

Figure 3. Alignment of NGAl, NRL and Uterocalin .
Protein sequences were obtained from the NCBI website. The alignment were done with the software of Vector NTI .

1.4.2 Lcn2 Ligands and Receptor

The barrel shape of the lipocalin easily leads to the idea that it has binding capability. Many proposed ligands of Lcn2 have been reported. Uterocalin can form a complex with an N-formyl peptide (FMLF) with a micro molar K_D. The same group showed that uterocalin can bind cholesteryl oleate, oleic acid, retinol, or retinoic acid at pH 7.4 with micro molar association constants. Uterocalin was reported to undergo a considerable conformation change in forming the protein-ligand complex (Chu et al., 1997; Chu et al., 1998). However, others found that fMLF and other proposed fatty acid ligands do not bind to NGAL (Bratt et al., 1999; Goetz et al., 2000). FMLF was also shown not to fit into the n-capric acid (NCA) binding site in the NGAL calyx (Goetz et al., 2000). NCA co-purified with NGAL but it was found that the shape and chemical properties of NCA did not match the NGAL binding pocket, which makes it unlikely to be a NGAL ligand. This group proposed that a macromolecule, such as a small protein, might be the preferred NGAL ligand because the NGAL calyx is large enough (Goetz et al., 2000; Kjeldsen et al., 2000). Later, they identified the phenolate/catecholate-type enterobacterial ferric siderophore (enterobactin, ENT), a low molecular weight and ferric-specific chelator, as a ligand of NGAL (Goetz et al., 2002). The catecholate-type siderophore is one of the two types of siderophores secreted by bacteria (the other type is aerobactin, a hydroxamate-type siderophore). Enterobactin has the highest affinity for Fe^{3+} ($K_D=10^{-49}$ M). The binding of NGAL and the Fe^{3+} enterobactin is tight and specific, with a K_D of 0.41 ± 0.11 nM The stoichiometry of NGAL:siderophore:Fe is 1:1:1 (Goetz et al., 2002). NGAL also binds carboxymycobactins, which are soluble siderophores of mycobacteria (Holmes et al., 2005). Uterocalin also binds enterobactin with a K_D of 0.43 nM as measured by a fluorescence quenching assay. This is consistent with its structure and high degree of sequence identity to NGAL (Fischbach et al., 2006; Flo et al., 2004).

Chicken Lcn2 was named EX-FABP because it was reported as the first extracellular protein that has the ability to selectively bind and transport fatty acids in extracellular fluids and blood. EX-FABP binds long-chain unsaturated fatty acids, such as oleic, linoleic, and arachidonic acid, with 0.2 nM dissociation constant (Cancedda et al., 1996).

It was long suspected that there are receptor(s) for Lcn2 on the cell surface. Megalin, which belongs to low density lipoprotein receptor family, was identified as a NGAL receptor

(Hvidberg et al., 2005). Megalin is one of the largest human proteins. As a type I transmembrane protein, it has a single membrane-crossing region and has 4398 amino acid residues in its extracellular region. The extracellular region has 36 low density lipoprotein receptor ligand binding repeats. Its cytoplasmic domain contains two signals for coated pit-mediated internalization and SH2 and SH3 recognition sites. Megalin binds apo-NGAL (NGAL without siderophore) with high affinity (K_D=~60 nM), and the LDL-receptor type-A repeats of megalin are involved in the binding. The siderophore–bound NGAL has a similar affinity with megalin (Flower, 2000; Hvidberg et al., 2005). Devireddy *et al.* identified a potential uterocalin receptor with 12 putative transmembrane helices that mediates cell internalization of uterocalin. This receptor has two forms: long form (60 kDa) and short form (30 kDa) due to alternative splicing. The long form of the receptor is widely expressed in murine tissues. However, no purified protein, western blot, or binding data of uterocalin with the receptor was reported (Devireddy et al., 2005).

1.4.3 Lcn2 Expression and Regulation

Lcn2 is expressed in many tissues. In the mouse, basal level uterocalin expression is high in the mammary gland and the uterus. Uterocalin expression in the mouse uterus is mainly in the luminal and glandular epithelial cells of the endometrium (Huang et al., 1999; Ryon et al., 2002). The expression of uterocalin is even higher in both mammary gland and uterus during involution. Ryon *et al.* estimated that 0.2 to 0.5% of the total extractable protein from the mammary gland and uterus is uterocalin (Ryon et al., 2002). The expression of uterocalin is also detected in mouse liver, spleen, epididymis, testis and lung (Chu et al., 2000; Garay-Rojas et al., 1996; Liu and Nilsen-Hamilton, 1995).

In rats, high expression of Lcn2 was detected in Neu-induced breast cancer, which is the reason for its being named NRL (Stoesz and Gould, 1995). NRL also shows high expression in prehypertrophic chondrocytes in rat embryos. By using immunohistochemistry, NRL was localized to the forming skeletal muscle fibers and to the myocardium of the developing heart (Zerega et al., 2000).

In the human, NGAL expression is detected in the normal human mammary gland and shows a heterogeneous expression pattern in primary breast cancer patients. However,

NGAL expression in human breast cancer is not associated with HER2/neu activation (Stoesz et al., 1998). In the bone marrow cells of myelocytes/metamylocytes, NGAL is synthesized during a narrow window of maturation of granulocyte precursors. The authors proposed and provided evidence that the reason for NGAL targeting to the secondary granule is the timing of NGAL biosynthesis rather than individual sorting information presented by the protein (Borregaard et al., 1995; Le Cabec et al., 1996). NGAL is almost undetectable in normal colon epithelial cells. But its expression is up-regulated in colon epithelial cells in colorectal neoplasia or inflammatory diseases (Nielsen et al., 1996). NGAL expression is also detected in normal lung, trachea and other tissues (Cowland and Borregaard, 1997).

EX-FABP was also suggested as a stress protein with physiological expression in tissues with active remodeling or in tissues with an acute phase response due to a pathological condition (Cermelli et al., 2000; Fiorella Descalzi et al., 2002).

Lcn2 expression is regulated by growth factors, hormones and cytokines. In mouse, uterocalin expression in uterus is regulated by ovarian steroids during the natural estrous cycle with a much higher expression in the proestrus and estrus stages (Huang et al., 1999). In Balb/c 3T3 cells, uterocalin expression is induced by bFGF and EGF (Davis et al., 1991a; Nilsen-Hamilton et al., 1982). TNFα alone can induce uterocalin expression in Balb/c normal liver (BNL) cells, and there is a synergistic effect of uterocalin expression in BNL cells when the cells are treated with TNFα together with IL-6 (Liu and Nilsen-Hamilton, 1995). However, in A549 cells, NGAL expression is upregulated by IL-1β but not by TNFα (Cowland et al., 2003). NRL expression in rat hepatocytes is upregulated by IL-1β but not by IL-6 (Jayaraman et al., 2005). IGF-I and G-CSF induce a marked increase in expression of uterocalin in myeloid cells (32D and derived cells) (Liu et al., 2005). But in FL5.12 cells, the addition of IGF-I to the culture medium blocks the high uterocalin expression caused by IL-3 deprivation (Devireddy et al., 2001). The different effects of IGF-I and other growth factors on uterocalin expression may due to the different cell types used in these experiments.

Some transcription factor binding sites were identified in the Lcn2 promoter that are important to the regulation of its expression. For example, two glucocorticoid responsive core elements (GRE) were found in the 5'-flanking region in the uterocalin gene, and

glucocorticoids (dexamethasone, Dex) induce the expression of uterocalin dramatically (Garay-Rojas et al., 1996).

A NF-κB site was identified as the important element in IL-1β induction of NGAL expression in A549 cells. Although TNFα also activates NF-κB, it alone did not induce NGAL expression in A549 cells, although it activated the expression of IL-8, a known target of TNFα and this function was used as positive control in the experiment. The authors proposed that IL-1β may induce or modulate another transcription factor besides NF-κB, which is important for activation of the NGAL promoter (Cowland et al., 2003).

Regulation of uterocalin expression by cytokines has also been reported. Uterocalin expression was induced by IL-9 in murine T lymphomas and STAT3 was believed to be involved in the process (Orabona et al., 2001). In 32D derived cell lines, uterocalin expression is upregulated through a G-CSF activated signal transduction pathway. STAT3 is activated by G-CSFR in the process and is an important mediator of increased uterocalin expression (Wang et al., 2005). C/EBPα plays a critical role in STAT3 regulation of uterocalin expression (Numata et al., 2005; Wang et al., 2005). Uterocalin was also identified as a novel target of IL-17. Uterocalin is regulated synergistically by IL-17 and TNFα in a preosteoblast cell line MC3T3-E1 (Shen et al., 2005). The target elements of IL-17 in uterocalin transcriptional regulation are a NFκB site and a C/EBP site (Shen et al., 2006). Other C/EBPs are also important in NGAL expression. C/EBPε was demonstrated to play an important role in the sorting of NGAL in neutrophil secondary granules (Gombart et al., 2003). In neuroblastoma cells, the uterocalin gene is a downstream target of C/EBPβ (Cortes-Canteli et al., 2004). All these reports support that STAT3, C/EBPs and NFκB are important in the regulation of uterocalin expression.

Other signal transduction pathways were also suggested to be involved in Lcn2 expression regulation. A recent paper reported two independent pathways that regulate uterocalin expression: the Wnt pathway and iron. Activation of the Wnt path way led to decreased expression of Lcn2. The iron concentration in the cell did not change during this process. On the other hand, down-regulation of uterocalin expression was observed when iron was depleted from the cell culture medium. Uterocalin expression was induced when iron was added to the iron-deprived cells (Ziegler et al., 2007). Others showed that p38, a

member of the MAP kinase superfamily, mediates uterocalin expression in the chondrocyte MC615 cell line and that NFκB is activated in the process (Ulivi et al., 2006).

1.4.4 Iron Delivery and Apoptosis

Iron is important for the growth of bacteria. One of the strategies of bacteria to obtain their iron supply is to synthesize a wide variety of low-molecular-weight iron chelators called siderophores, which are synthesized, secreted and taken up again by many microorganisms when the microorganisms can not obtain sufficient soluble iron from their environment directly (Braun and Killmann, 1999). Enterobactin, a catecholate-type siderophore from *E. coli*, has the highest affinity for ferric iron. Lcn2 binds enterobactin after the body is invaded by *E. coli* and prevents *E. coli* from obtaining iron (Flo et al., 2004). Thus, Lcn2 is proposed as part of the innate immune system and works as a bacteriostatic agent *via* an antibacterial iron depletion strategy (Goetz et al., 2002). This hypothesis is supported by in *vivo* experiments, in which Lcn2 knockout mice showed much lower survival rates than wild type mice when injected with a strain of *E coli* that acquires iron from the host in an enterobactin-dependent way. But the responses of Lcn2 deficient mice and wild-type mice were the same when they were injected with bacteria whose siderophore does not bind Lcn2 (Flo et al., 2004).

Kidney ischemia-reperfusion injury induces acute tubular necrosis (ATN) in the human. During this process, a significantly increased level of NGAL is detected in the serum and the urine. Uterocalin expression increases extensively in the mouse kidney ischemia-reperfusion injury model and the injection of exogenous iron-loaded uterocalin (uterocalin:siderophore:Fe complex), but not uterocalin:siderophore complex or uterocalin itself, rescues the mouse proximal tubule from ATN. This indicates that the delivery of iron by uterocalin is the essential factor for the rescue. An iron-binding cofactor was also reported in urine samples from normal mice (Mori et al., 2005). Berger *et al.* also showed the Lcn2 knock-out mice are more sensitive than wild type mice to *E. coli* infection, but they did not find that Lcn2 deficient mice showed any difference from wild type mice in their response to ischemia reperfusion injury (Berger et al., 2006). Lcn2 expression was detected in ureteric bud cells and it induces mesenchymal to epithelial conversion. The authors proposed that this

is caused by the ability of Lcn2 to bind and deliver iron to the cytoplasm, where iron activates or represses iron-responsive genes, in a pathway that differs from that of transferrin (Yang et al., 2002). Lcn2 delivery of ferric iron to mouse spermatozoa is also reported (Elangovan et al., 2004).

Several papers reported that Lcn2 is involved in apoptosis as a proapoptotic factor. For example, uterocalin induced apoptosis in FL5.12 cells (Devireddy et al., 2001). IL-3 is very important for the culture of FL5.12 pro-B cells. When IL-3 was deprived from the culture medium, FL5.12 pro-B cells underwent apoptosis. Increased uterocalin transcription was associated with apoptosis. Conditioned medium containing uterocalin induced apoptosis in FL5.12 pro-B cell line and other cell lines. Recombinant uterocalin from *E. coli* also has this ability. Moreover, uterocalin antisense RNA or antibody can block this effect. All these results lead to the conclusion that uterocalin can induce apoptosis in certain cell types (Devireddy et al., 2001). The relation of Lcn2 and apoptosis has also been reported by other groups. Bong *et al.* reported that transient expression of uterocalin induces apoptosis in HC11 cell line, which is a mouse mammary epithelial cell line (Bong et al., 2004). High expression of uterocalin is detected in mouse erythroid progenitor cells and uterocalin induces apoptosis and inhibits differentiation of erythroid progenitor cells in cell culture experiments (Miharada et al., 2005). Mouse Bcr-Abl positive hematopoietic cells persistently express and secrete uterocalin, which induces apoptosis in Bcr-Abl negative but not Bcr-Abl positive cells (Lin et al., 2005). Lcn2 is secreted by inflammatory activated microglia and renders microglia more sensitive to apoptotic signals in an autocrine manner (Lee et al., 2007).

Lcn2 is also reported as a survival factor. MK886, a strong proapoptotic agent, simultaneous induces apoptosis in FL5.12 cells and uterocalin mRNA expression. It was assumed at first that the increased expression of uterocalin is an apoptotic mechanism of MK886. But later, the same group suggested that NGAL might be actually a survival factor because overexpression of NGAL protects cells from apoptosis induced by phosphoinositide-dependent kinase 1 (PDK1) inhibitors and NGAL antisera induces apoptosis in human MCF7 and A549 cell lines with stable NGAL overexpression (Tong et al., 2003; Tong et al., 2005).

The mechanism of Lcn2 induced apoptosis remains unclear. In 2005, Devireddy *et al.* (Devireddy et al., 2005) proposed a hypothesis that links Lcn2 induced apoptosis and iron delivery. In the hypothesis, an Lcn2 cell membrane receptor mediates the intake of Lcn2. In the cell, there is proposed to exist a siderophore-like molecule that binds iron with high affinity. Once inside the cell, Lcn2 binds the siderophore-iron complex. The subsequent export of Lcn2-siderophore-iron out of the cell leads to iron depletion, which causes the cell to undergo apoptosis. They cloned a proposed receptor of Lcn2 (24p3R) and overexpressed it in Hela cells. They found the 24p3R expression promotes Lcn2 uptake by 24p3R cells. They also reported that both iron loaded Lcn2 (holo-Lcn2) and iron unloaded Lcn2 (apo-Lcn2) can be taken up by cells expressing 24p3R, but the apo-Lcn2 specially mediates intracellular iron depletion and apoptosis. These results support the hypothesis. However, they did not show the protein binding of the 24p3R and Lcn2, and the endogenous siderophore like molecule is not found yet (Devireddy et al., 2005).

Recently, EX-FABP was proposed as a cell survival factor. In one paper, Ex-FABP expression was down-regulated by simultaneous expression of EX-FABP antisense RNA in chicken embryo chondrocytes. The researchers observed an increase in the number of apoptotic chondrocytes. The expression of antisense RNA to EX-FABP also induced extensive myoblast cell death (Eddi Di Marco, 2003). Another paper reported that EX-FABP is diffusely expressed in chicken embryos at 3.5 days of development. Direct microinjection of EX-FABP antibody into chicken embryos induced significant cell apoptosis in embryo hearts, which eventually led to embryonic death (up to 70% of embryos died). The authors suggested that the role of EX-FABP as a scavenger for fatty acids is the reason that it promotes cell survival (C. Gentili, 2005).

1.4.5 Cancer and Other Possible Functions

In the rat, NRL expression is associated with breast cancer induced by neu. However, this is not true for human beings. The relationship of Lcn2 and breast cancer is not clear. NGAL expression in breast cancer is heterogeneous: some patients samples have NGAL expression while some do not and some are ambiguous. It was once suggested as a putative *in vivo* estrogen target gene and a new therapeutic target gene for breast cancer prevention

and treatment as in a mouse model (Seth et al., 2002). But no other reports support this opinion.

NGAL may play a role in breast cancer growth. The complex of NGAL and gelatinase B (MMP-9), which is found in human neutrophils, can form *in vitro* by mixing NGAL and MMP-9. It appears that, when complexed with MMP-9, NGAL can protect the MMP-9 from degradation and preserve the MMP-9 enzymatic activity (Yan et al., 2001). One group reported that NGAL overexpression stimulates MCF-7 breast cancer cells growth in immune-deficient mice, and a higher level of MMP-9 was detected in NGAL-overexpressing tumors compared to the wild type tumor. The explanation is that protection of MMP-9 activity facilitates tumor angiogenesis and tumor growth. The NGAL/MMP-9 complex is present in the urine of breast cancer patients, and detection of the complex may be used as a noninvasive method for predicting disease status of breast cancer patients (Fernandez et al., 2005). However, protection of MMP-9 is probably unique to NGAL, because NGAL is the only Lcn2 ortholog with a free Cys residue and forms a complex with MMP-9, while uterocalin and NRL do not form the complex with MMP-9 because they do not have the third free Cys residue.

Lcn2 suppression of cancer cell invasiveness and metastasis has been reported (Hanai et al., 2005). The metastatic potential of the breast cancer model (4T1 cell line) was accelerated by the introduction of an active mouse H-Ras mutant. Overexpression of uterocalin converted the 4T1-Ras-transformed tumor cells to an epithelial phenotype with an up-regulation of E-cadherin expression. Suppression of cell invasiveness in cell culture and tumor growth and lung metastases *in vivo* was also observed (Hanai et al., 2005). Others reported suppression of the invasion and liver metastasis of colon cancer cells with expression of NGAL (Lee et al., 2006). NGAL expression was also found in other tumor cell lines and tumor tissues, such as in pancreatic tumors and pancreatic cell lines. NGAL expression in colorectal and hepatic tumor tissues is significantly higher than in normal tissues. But the function of NGAL expression in these tumor tissues is not clear (Furutani et al., 1998).

There are other proposed characteristics or functions of Lcn2. For example, NGAL induced cell migration was reported. NGAL can stimulate mouse inner medullary collecting

duct (mIMCD)-3 cell migration and was required for efficient mIMCD-3 cells tubulogenesis in cell culture (Gwira et al., 2005). Uterocalin increased cell migration in cell culture and facilitated rat mucosal regeneration by promoting cell migration *in vivo via* subcutaneous administration (Playford et al., 2006). Uterocalin is associated with sperm mobility or activity. Addition of uterocalin enhanced sperm progressive motility but not hyper-activated movement. This implies that uterocalin may play a role in flagellar motility regulation (Lee et al., 2003). Uterocalin has the ability to inhibit the acrosome reaction in BSA stimulated sperm *in vitro* (Lee et al., 2005). Uterocalin can be phosphorylated by PKC *in vitro* and the phosphorylated form of uterocalin was detected in the mouse uterus (Lee et al., 2001).

1.4.6 Biomarker for Diseases

NGAL is in the secondary granules of neutrophils and is secreted during neutrophil degranulation. Thus, its serum concentration may reflect granulocyte activity and can be used as a marker for some diseases. NGAL was one of biomarkers used to evaluate granulocyte activation in atherosclerosis (Elneihoum et al., 1997). It was suggested as an even more sensitive and specific diagnostic tool than myeloperoxidase (MPO) to monitor neutrophilic inflammation in cystic fibrosis (Eichler et al., 1999). Both NGAL and MPO can be detected in lung lavage fluid and lung tissue. NGAL was suggested as a better marker to measure neutrophil cell activity within the airspace than MPO because its concentration is less variable (Schmekel et al., 2000). NGAL, mostly in the dimeric form, increased more than 4 fold in the plasma after cardiac surgery. The release of NGAL was suggested as a result of inflammatory activation of neutrophils caused by the extracorporeal circulation (ECC) used during cardiac surgery (Jonsson et al., 1999). In ulcerative colitis, a significant increase in NGAL was detected in rectal dialyzates and feces but not in the serum (Nielsen et al., 1999). NGAL was suggested as a predictor for the long-term mortality after cerebrovascular ischemia, because it indicates leucocyte activation (Falke et al., 2000).

NGAL expresses in the epidermis but solely within the hair follicles in normal adult skin. However, NGAL expression is strongly induced in the epidermis in skin disorders that are characterized by dysregulated epithelial differentiation. The confined NGAL expression in adult parakeratotic epidermis makes it a marker for dysregulated keratinocyte

differentiation in human skin (Mallbris et al., 2002). NGAL expression in Kawasaki disease is higher than normal for a long time after onset of the disease as a result of sustained neutrophil activation (Biezeveld et al., 2005).

NGAL is suggested as a useful marker in the diagnosis of acute bacterial infections because the serum NGAL level increases only in acute bacterial but not in viral infection (Xu et al., 1995). It was also selected as a useful early marker of neonatal bacterial infection because NGAL is rapidly released by neonatal neutrophils upon microbial stimulation *in vivo* (Bjorkqvist et al., 2004). In the mouse and rat models, Lcn2 expression is highly increased in the kidney and can be easily detected in urine after ischemic renal injury. The increase of NGAL even precedes the increase of traditional markers such as β-2 microglobulin and N-acetyl-beta-D-glucosaminidase (NAG). The rapid increase of NGAL after ischemia makes it an early urinary biomarker for ischemic renal injury (Mishra et al., 2003).

1.5 Human Breast Development

The breast is an important and complex organ for the nursing and survival of the newborn. There are two tissue compartments, the epithelium and the stroma, that constitute the mammary gland. The epithelial cells form ducts and milk-producing alveolar cells and the stromal cells form the mammary fat pad. Human breast development starts during embryonic life. Throughout early life, the breast undergoes dramatic size, shape, and functional changes in association with puberty, pregnancy, lactation, weaning and postmenopausal regression (Vorherr, 1974b). The breast remains relatively quiescent after birth until puberty. At puberty, the breast grows fast with lobule formation, and gains its mature shape after puberty. However, breast development and differentiation persist until the end of the first full term pregnancy. The breast attains its maximum development at the end of pregnancy, secretes milk during lactation and involutes after weaning. The breast regresses after menopause (Russo and Russo, 2004).

1.5.1 Prenatal, Perinatal and Infant Breast Development

The mammary gland parenchyma is derived from the ectoderm around the fifth week of gestation in the human. The mammary ridge, a 4 to 6 layer epithelial band in the thoracic

area forms during the sixth and seventh weeks of gestation, becoming the mammary gland. Because of the variation in the nomenclature and the difficulty in establishing the exact time of conception, it is difficult for authors to agree on the exact time of appearance of each structure. The Russos consider that correlation of the phases of mammary gland development with embryonic or fetal length is a more accurate method for describing prenatal breast development. They constructed a table in which they divided the mammary gland development during gestation into 10 different stages [Table 1] (Russo and Russo, 2004; Vorherr, 1974b).

Stage	*Mammary gland development*	**Embryo-fetal stage**
1	Ridge stage	Less than 5mm embryo
2	Milk hill stage	More than 5.5-mm embryo
3	Mammary disc stage	Around 10–11mm embryo
4	Lobule type stage	11.0–25.0-mm embryo
5	Cone stage	25–30-mm embryo
6	Budding stag	30–68-mm embryo
7	Indentation stage	68-mm to 10 cm
8	Branching stage	10 cm fetus
9	Canalization stage	20 and 32 weeks of gestation
10	End-vesicle stage, in which the end vesicles are composed of a monolayer of epithelium and contain colostrums	Newborn

Table 1. Stages of prenatal development of the human breast. (Reprinted from (Goetz et al., 2002), Copyright (2004), with permission from Elsevier)

At birth, the mammary discs may be observed in neonates of both girls and boys because of stimulation of fetal mammary gland tissues by the maternal luteal and placental sex steroids (Vorherr, 1974b). The newborn breast contains very primitive structures that are composed of short ductules. The ductules are lined by one to two epithelial cell layers and one myoepithelial cell layer. The morphological structures of the breasts in boys and girls are similar from birth to puberty. However, the variation in the degree of glandular development (branching and acinar formation) and the functional differentiation of the cells lining the ducts and acini is huge between individuals, even between breasts of the same individual.

Beatrice *et al*. (Beatrice and Barry, 2000) proposed a system to represent the prepubertal breast. In their system, three morphological types describe the degree of glandular development and branching complexity and five functional stages describe the secretory activity and glandular involution. Various combinations of morphological types and functional stages were observed and used to describe the breast. However, there is no clear correlation between the morphological types and the functional stages (Beatrice and Barry, 2000; Russo and Russo, 2004).

The cells forming the epithelial compartment of the mammary gland were proposed to be derived from mammary stem cells (MSCs). MSCs have the capacity to renew themselves and give rise to committed epithelial precursor cells (EPCs). The progeny of EPCs are destined to one of two fates. One is the ductal fate, in which they become ductal precursor cells (DPs) that form ducts. The other is the alveolar fate, in which they become alveolar precursor cells (APs) that give rise to differentiated milk-producing cells (Hennighausen and Robinson, 2005). That a small section of transplanted mammary gland duct can generate an entire ductal tree in a cleared fat pad is evidence of the presence of the stem cells (Kordon and Smith, 1998). Most epithelial cells in postnatal mammary tissue express estrogen and progesterone receptors, and the infant breast is able to respond to maternal hormones. This is the reason for the generalized secretory activity of the newborn gland (Keeling et al., 2000). However, the differentiated infant breast undergoes involution once the influence of the maternal hormones has faded. The infant breast contains only small ductal structures in a fibroblastic stroma and remains in this state until puberty (Beatrice and Barry, 2000; Jean W. Keeling, 2000; Russo and Russo, 2004).

1.5.2 Pubertal Development of the Human Breast

The breast initiates its development at puberty. An early sign of breast development is increased tissue succulence and visible elevation of areola mammae and the nipple. The first recognizable enlargement of the breast disc usually occurs at 10-years of age. Changes of the breast in form and size during this period may last for 2-3 years. This adolescent period terminates with sexual maturity (Jose Russo, 2000; Vorherr, 1974b).

The gross change in the breast at puberty is caused by both epithelial and stromal growth. The amount of fibrous and fatty tissue increases in the stroma and the stromal tissue functions as a matrix for the growth of the ducts (Beatrice and Barry, 2000). The growth and division of the primary ducts, which are a combination of dichotomous branching ducts (dividing into two parts) and sympodial branching ducts (forming an apparent main axis from successive secondary axes), contribute to the increase of the parenchyma of the breast (Russo, 1996; Russo J, 1982).

Ductal elongation and formation of lobular structures in the female breast during puberty is under the influence of hormones (Beatrice and Barry, 2000). Ovarian hormones, especially estrogens and progesterone, are important to the growth of mammary ducts. But in early puberty there is only a small amount of progesterone available because ovulation of the primordial follicles is absent. Hence, the major inductive factor of breast development is estrogens in this period. Later during puberty, with ovulation of the ovary, progesterone is available, and breast development occurs under the influence of both estrogens and progesterone (Vorherr, 1974b).

Growth hormone (GH) also plays a role in breast development. GH directly stimulates ductal tree elongation and branching during puberty, although the exact mechanism of regulation is unclear. In hypophysectomized–ovariectomized rats, ductal growth is under the direct influence of GH. This effect of GH on breast development might work through its local mediator, insulin-like growth factor I (Jose Russo, 2000).

The main morphological structure of the breast during puberty is a cluster of approximately 11 alveolar buds around a terminal duct. In the morphological classification of Dr. Russo, the structure is named as type 1 (Lob 1) or virginal lobule. Alveolar buds originate from the club-shaped terminal end buds (TEB), which result from dichotomous and sympodial division of the primary ducts. Each TEB divides into two alveolar buds. Alveolar buds become smaller with further branching and form ductules. The lining of the TEB and ductules are two-layered epithelia [Fig 4, lobular structure] (Jose Russo, 2000; Russo and Russo, 2004).

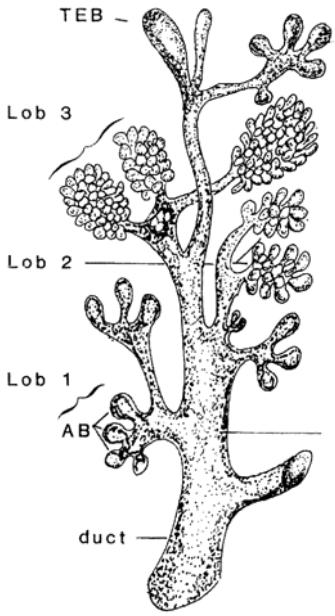

Fig. 4

Figure 4. Diagrammatic representation of the lobular structures of the human breast. (Reprinted from (Goetz et al., 2002), Copyright (2004), with permission from Elsevier)

1.5.3 The Adult Human Breast

The shape of the breast is determined mainly by connective tissue growth and fat deposition under the influence of hormones. The breast usually obtains its definite adult shape after the age of 15 (Vorherr, 1974b). Development of the epithelial tissue persists after the breast gains its mature shape. Besides the type 1 lobule, two other identifiable types of lobules can be found in the normal breast tissue of adult women. They are designated as type 2 (Lob 2) and type 3 (Lob 3) lobule. Lob 2 and Lob 3 are the result of further differentiation of Lob 1. The alveolar buds of Lob 1 sprout new ductules to form Lob 2 and Lob 3 with the result that there are more ductules in Lob 2 and Lob 3 than in Lob 1. There are approximately 47 and 80 ductules in Lob 2 and 3 respectively, but there are only 11 ductules in Lob 1. During the transition from Lob 1 to Lob 2, or Lob 2 to Lob 3, the lobule size enlarges.

However, the size of the individual ductule structure, which forms the lobule, is reduced (Jose Russo, 2000; Russo and Russo, 2004).

Although all three types of lobule can be observed in the nulliparous breast, their distributions differ. Lob 1 is the predominate structure in the nulliparous breast and remains constant with breast development. Lob 2 and Lob 3 can be seen in the nulliparous breast, but in much fewer numbers than Lob 1. However, huge variations exist in the breast structure among normal nulliparous women and even between the breasts of the same woman, which makes it very difficult to define states of the breast solely by the distribution of lobules (Beatrice and Barry, 2000; Jose Russo, 2000; Russo and Russo, 2004). Although the stromal tissue may accomplish its differentiation as early as the age of 14-15 years, the epithelial tissue maintains its developmental state for many years under the influence of cyclic ovarian sex hormones (Vorherr, 1974b).

1.5.4 Pregnancy and Lactating Human Breast

During pregnancy, the breast changes in appearance and structure, such as the intensification of the pigmentation of the areola and nipple and the enlargement of the areola mammae. The breast, especially the peripheral mammary areas, also enlarges. It is believed that the enlargement of breast during pregnancy is caused mostly by extensive ductular-lobular-alveolar growth. The growth is induced by multiple luteal and placental hormones, but the estrogens and progesterone are the most important hormones. During pregnancy, the fat and connective tissue decrease in proportion to the breast as a result of dilation of the alveoli, colostrum accumulation and vascularization enhancement (Vorherr, 1974b).

Two morphological phases are observed in pregnant breast development. The first phase starts as early as 3 to 4 weeks of gestation and is characterized by ductal lengthening and profuse branching. As a result of the rapid formation of ductules, more Lob 2 and Lob 3 are formed. Besides the hypertrophy of preexisting structures, new lobular formation also exists in the breast. The second phase starts at the end of the third month when alveoli begin to show secretory function. At this phase, a new type of lobule, the Lobule type 4 (Lob 4) presents in the breast. Lob 4, found only in parous women and not in nulliparous women, is a fully differentiated structure and has characteristic single cell layered secretory acini. The

epithelial cells of the acini increase greatly in number due to active cell division and they also increase in size mainly by cytoplasmic enlargement (Russo et al., 2005; Russo and Russo, 2004; Vorherr, 1974b).

Development of the ductal tree is accomplished by the end of the first half of pregnancy and the breast gains its secretory function. The second half of gestation is characterized by continuation and accentuation of the secretory activity with only minor additional branching. The formation of differentiated secreting acini becomes more evident during this period. At this time, the epithelial cells are highly differentiated and only show minimal proliferative activity. Colostrum, secretory material composed of desquamated glandular and phagocytic cells, begins to fill the already formed luminae. The secretion of colostrum augments as the pregnancy advances. The breast becomes firm and full and ready for lactation at term (Russo and Russo, 2004; Vorherr, 1974b).

The morphological changes of the mammary gland during lactation are minor with the observation of enlargement of lobules and dilation of acini. However, the alveolar cells, under the influence of a neuroendocrine control, achieve a conversion from the presecretory stage into an active milk protein synthesizing and releasing stage. Lactogenesis is the synthesis and release of milk into the mammary acini and ductal system. It is induced mainly by the combination of a high level of prolactin and a rapid withdrawel of estrogens, progesterones and placental lactogen after parturition. Colostrum, which is rich in minerals and immune factors and low in lactos, is secreted in the first 3-5 days. Secretion of mature milk, which has a higher concentration of lactose and a lower concentration of sodium and chloride, starts to be evident in the mother by day 10 of lactation. The initiation of lactogenesis does not require suckling of the infant, but it is important to maintain lactation because the removal of milk from the breast is critical for the alveolar cells to maintain the ability of milk secretion. Milk can be stored for up to 48 hours in the acini and ductal system before the reduction of milk secretion begins. If the milk is removed regularly from the mammary gland, lactation of women can be maintained for up to 4 years (Picciano, 2003; Russo et al., 2005; Vorherr, 1974b).

1.5.5 Postlactational Involution

When milk removal is discontinued, prolactin secretion decreases and mammary gland involution ensues. However, postlactational involution is mostly because of the inhibitory effect of the milk stored in the mammary gland ductal system. Milk storage causes alveolar distention and tissue hypoxia and eventually inhibits further milk synthesis. Two complementary mechanisms are responsible for postlactational mammary gland tissue regression. One is cell autolysis because of reduced alveolar oxygen and nutrient supply. The other factor is the subsequent round of phagocytic cell infiltration and phagocytosis of the degenerated and necrotic alveolar material. During this process, the cellular borders disappear, the acinar structures collapse, and the tubules become narrow. But the ducts show little change in this process. All these changes lead to the reduction of the lobular-glandular element. New formation of connective tissue follows parenchymal reduction. The involutional process may take 3 months in the human and eventually the two layered epithelia of the resting mammary gland are reformed. However, the breast structure after involution is not the same as the nulliparous breast. The involuted breast retains more glandular tissue (mainly Lob3) compared to nulliparous breast (mainly Lob1). The structural changes of the breast will repeat in successive rounds of pregnancy and involution (Beatrice and Barry, 2000; Russo et al., 2001; Russo et al., 2005; Russo and Russo, 2004; Vorherr, 1974a).

1.5.6 Menopausal Involution

Ovarian hormones have an important effect on development of the breast. However, the production of ovarian steroids in women decreases during the perimenopause period to almost complete cessation after menopause, which is characterized by amenorrhea. With the decrease and cessation of production of ovarian steroids, the breast undergoes involution, both in parous and nulliparous breasts. Menopausal involution is different from postlactational involution because lobules and ducts are both reduced in number during menopausal involution whereas ducts show little change during postlactational involution. Menopausal regression of the mammary gland starts from the glandular epithelium and parts of the connective tissue disappear along with it. As a result of regression, the numbers of

Lob2 and Lob3 are reduced and the number of Lob1 is augmented. At the end of the fifth decade of life, the histological appearance of the breast of nulliparous and parous women is almost identical and both contain predominantly Lob1 structures. Because premenopausal parous women have more Lob2 and Lob3 in their breasts than in breasts of nulliparous women, their regression is different in a qualitative aspect although the results of involution are the same. During menopausal involution, fat is deposited in the breast and replaces the glandular epithelium and the connective tissue. Only a few acini and ducts remain after involution (Beatrice and Barry, 2000; Russo et al., 1992; Russo and Russo, 2004; Vorherr, 1974a).

1.5.7 Pregnancy and Breast Cancer

Breast cancer is the major cancer afflicting women of western world countries. It is estimated that more than ten percent of women in the U.S. have a lifetime risk of developing breast cancer. After lung and bronchial cancer, breast cancer is the third leading cause of cancer deaths in the U.S. (Jemal et al., 2003).

Reproductive history, such as early menarche, menopause and parity, is a consistent risk factor among many others for breast cancer. However, one of the strongest protective factors for breast cancer is also related to reproductive history: a full term pregnancy early in life (early 20s) gives a 50% reduction of lifetime risk of breast cancer when compared with the risk in nulliparous women. Although additional pregnancies further increase the protective effect, the greatest protection is from the first early full term pregnancy. The protective effect of a full term pregnancy early in life has been demonstrated in many epidemiological studies and is considered by some researchers as a practical and affordable way for prevention of breast cancer in humans (Bernstein, 2002; Lambe et al., 1996; MacMahon et al., 1970).

The protective effect of an early full-term pregnancy against breast cancer is also observed in experimental rodent models. Rats and mice have long served as animal models for chemically induced breast cancer research. Rats and mice that have undergone a full term pregnancy also gain protection against chemically induced mammary carcinogenesis when compared with nulliparous animals (Russo and Russo, 1996; Sinha et al., 1988; Welsch,

1985). Although most published papers report the use of rats in these experiments, the fully developed technology for preparing transgenic and knockout mice will provide an advantage to using mice in breast cancer studies.

Ovarian steroid hormonal changes during pregnancy have been suggested as an explanation for the protective effect of pregnancy against breast cancer. The mammary gland is expected to have high levels of estrogens and progesterone during pregnancy. In the experimental rodent models, a short-term treatment with estrogen and progesterone to mimic the effect of pregnancy, prior to or after treatment with the chemical mammary carcinogen, also induced protection against breast cancer both in mice and rats (Guzman et al., 1999; Medina and Kittrell, 2003; Medina and Smith, 1999; Sivaraman et al., 1998; Yang et al., 1999). However, despite the well-documented data, the mechanisms of pregnancy/hormone-induced protection against breast cancer still remain unclear. Breast cancer incidence reaches its peak after menopause when the morphology of the breast is the virtually the same in parous and nulliparous women with Lob1 structure as the main epithelial structure, (Jemal et al., 2003). However, the nulliparous women are at higher risk of developing breast cancer while parous women are protected (Russo and Russo, 2004). Hypotheses to explain this effect include 1) pregnancy or hormone treatment caused differentiation of the mammary gland. Differentiation may remove a population of cancer susceptible cells (in the terminal end buds) and hence protect the mammary gland from cancer (Russo and Russo, 1997), 2) susceptibility of the mammary gland to cancer is modulated by the hormonal milieu, which results in persistent biochemical alterations in the mammary epithelia (Swanson et al., 1995), 3) the developmental fate of a subset of mammary epithelial cells is affected by the hormonal milieu of pregnancy and this increases protection against breast cancer (Medina and Kittrell, 2003; Swanson et al., 1995).

1.5.8 Macrophages and Mammary Gland Development

Macrophages are differentiated from blood monocytes, are recruited into tissues and can be found in every tissue (Gouon-Evans et al., 2000). They are given special names in some tissues such as osteoclasts (bone), microglia (brain), Kupffer cells (liver) and mesangial cells (kidney). Macrophages play an important role in host defense against pathogens and are

thought to participate in tissue remodeling during development and tissue homeostasis (Gouon-Evans et al., 2000).

Macrophages are also found in the mammary gland. They are involved in regulating ductal morphogenesis, which is an important aspect of postnatal mammary gland development. Macrophages are found mainly around the neck of the terminal end buds and not associated with the ductal structures distal to the terminal end buds during pubertal mammary gland development in the mouse (Gouon-Evans et al., 2000; Ingman et al., 2006). Depletion of the macrophage population from the mammary glands by whole body γ-irradiation or by knockout of Colony Stimulating Factor-1 (CSF1), which is required to recruit macrophages into mammary glands, results in delayed formation, reduced numbers and a shorter and rounder form of the terminal end buds compared to those in the control glands (Gouon-Evans et al., 2000; Ingman et al., 2006). The defects of the terminal end buds have been shown to be directly due to macrophage depletion because restoration of the macrophage population in the mammary gland rescues these defects (Gouon-Evans et al., 2000; Ingman et al., 2006). Thus, macrophages are required for ductal morphogenesis in postnatal mammary gland development.

Macrophages are also found dispersed throughout the adipose tissue of the mammary fat pad (Schwertfeger et al., 2006). The exact function of macrophages in the fat pad is not clear, but it is most likely that they are involved in phagocytosing dying adipocytes (Cinti et al., 2005). Phagocytosis performed by macrophages may also be important for mammary gland remodeling during involution because many cells undergo apoptosis and need to be removed (Li et al., 1997; Lund et al., 1996). Macrophages in the mammary gland help to clear residual milk and apoptotic cells, although the mammary gland epithelial cells are also capable of phagocytosis (Helminen and Ericsson, 1968; Helminen and Ericsson, 1971; Walker et al., 1989). Earlier studies showed no increase in the population size of macrophages in the mammary gland during involution (Mayberry, 1964; Richards and Benson, 1971). But, recent data from microarray and histological studies of involuting mammary glands provided evidence for there being more macrophages in the mammary gland during involution (Clarkson et al., 2004; Stein et al., 2004). The increase in macrophages in the mammary gland during involution is believed to be due to monocyte

infiltration and subsequent differentiation into macrophages (Stein et al., 2004). This increase of macrophages is proposed to help remove apoptotic cells (Hanayama and Nagata, 2005; Monks et al., 2002). But the fate of the macrophages that infiltrate into the mammary gland during involution is not clear. D'Cruz *et al* reported that the expression of two macrophage specifically expressed genes, macrophage expressed gene 1 and macrophage metalloelastase, increases in fully involuted parous mouse mammary glands compared to virgin mammary glands in their microarray study (D'Cruz et al., 2002). This study supports the hypothesis that some macrophages may stay in the mammary gland after completion of involution, although there is no immunohistological study to confirm this finding.

1.5.9 Macrophage and Cancer

That surveillance provided by the immune system, including macrophages, might suppress the initiation of cancer was proposed a long time ago (Burnet, 1957). In a recent model, three stages in cancer are proposed. These are immunoediting (destruction of tumor cells by the immune system), equilibrium (tumor cells gain the ability to survive an immune attack), escape (tumor cells grow and expand in the immunocompetent host) (Dunn et al., 2002). According to this model, macrophages, together with other immune cells such as NK cells, are recruited to the tumor site and destroy tumor cells during the elimination stage (Dunn et al., 2002).

Tumor-associated macrophages (TAMs) promote growth and spreading of tumor cells (Elgert et al., 1998; Lin et al., 2002). This presents a paradox in which macrophages can both destroy tumor cells and promote tumor development. The explanation of this apparently paradoxical role of macrophages on tumor cells is the functional plasticity of macrophages (Sica et al., 2008). Two subsets of macrophages have been defined based on the secretion of either IL-12 (M1) or IL-10 (M2) in response to inflammatory stimuli (Trinchieri, 2003). M1 macrophages are considered to function in killing microorganisms and tumor cells, while M2 macrophages are able to promote angiogenesis, tissue remodeling and repair (Mantovani et al., 2004). Macrophages can be phenotypically polarized by the microenvironment to have either M1 or M2 function. For example, the classic activation signals such as LPS and IFN-γ induce the differentiation of macrophages into M1 phenotype' while signals such as IL-10,

IL-3, IL-4 or glucocorticoid hormones induce the differentiation of macrophages into M2 phenotype (Gordon and Taylor, 2005; Mantovani et al., 2002). The phenotype of TAMs is similar to that of M2 macrophages (Sica and Bronte, 2007). Thus, an explanation for the paradoxical role of macrophages on tumor cells is the following: M1 phenotype macrophages can cause death of tumor cells so they function in the elimination stage of the three-stage model and help to decrease cancer incidence. However, tumor cells have the ability to form a microenvironment that promotes macrophages to convert to the M2 phenotype that can then promote the growth of tumor cells during the escape stage (Sica and Bronte, 2007; Sica et al., 2008).

1.6 Mycoplasma

Mycoplasmas are a heterogeneous group of prokaryotes. They are the smallest organisms, in both cellular dimensions and genome size, which are capable of self-replicating and cell-free existence (Wilson and Collier, 1976). The first mycoplasma was described as the bovine pleuropneumonia agent by Nocard and Roux in 1898, and the name pleuropneumonia-like organisms (PPLO) was used for a period of time (Waites and Talkington, 2004). Mycoplasmas are distinguished from other bacteria by their minute size (1 to 2 μm long and 0.1 to 0.2 μm wide), total lack of a cell wall, cholesterol requirement for survival, small genome size (580kb to 1350kb) and low GC content (23-40%) (Razin et al., 1998).

Mycoplasmas are widespread in the animal kingdom. More than 100 species of mycoplasma have been identified. The primary habitats of human and animal mycoplasmas are the mucous surfaces of the respiratory and urogenital tracts, mammary glands, joints and the eyes (Razin et al., 1998). Mycoplasmas live an obligate parasitic mode of life and are usually host-specific. However, some mycoplasmas have a broader host range. For example, *Mycoplasma bovis*, which can cause mastitis in cattle, is also found in human (Pitcher and Nicholas, 2005).

Mycoplasma infection is a common problem in cell cultures. It was estimated that 5-87% of cell lines are contaminated with mycoplasmas depending on the sensitivity of the

method used to detect the infection (Hay et al., 1989; Rawadi and Dussurget, 1995). The main source of mycoplasma contamination of clean cell cultures is believed to be previously infected cultures. Laboratory personnel and commercial sera used in culture media are less common sources in mycoplasma contamination (Hay et al., 1989). Among the more than 100 mycoplasma species, 5 species account for more than 95% of cell contaminations. They are *Mycoplasma arginini, Acholeplasma laidlawii, Mycoplasma orale*, *Mycoplasma fermentans* and *Mycoplasma hyorhinis* (Bolske, 1988; McGarrity et al., 1986; Ossewaarde et al., 1996; Rawadi and Dussurget, 1995).

Contamination of cell cultures by mycoplasmas is more difficult to notice than contamination by bacteria and fungi because they can not be visualized under an inverted microscope and the medium is not turbid even though the concentration of mycoplasmas in the medium can be as high as 10^7-10^8 colony forming units (cfu) per milliliter. Mycoplasmas also usually exert no cytopathogenic effects on the host cells. In addition, mycoplasmas are resistant to the antibiotics commonly used in long-term cell cultures (such as penicillin) (Hay et al., 1989; Rawadi and Dussurget, 1995; Uphoff et al., 1992). All these reasons make periodic screening for mycoplasmas an important procedure to control contamination in cell cultures.

Many methods are used to detect mycoplasma in cell cultures such as microbial culture, DNA fluorochrome staining, immunofluorescence, biomedical assay and others. But currently, PCR is the preferred method to detect mycoplasma specific genes for screening because it is easy, fast and sensitive (Gopalkrishna et al., 2007; Kong et al., 2007; Rawadi and Dussurget, 1995; Uphoff et al., 1992).

Although mycoplasmas in contaminated cell cultures usually exert no obvious damage to the cells, they are capable of affecting the properties of cultured cells such as their growth, morphology and physiology (Doersen and Stanbridge, 1981; Hay et al., 1989; Rawadi and Dussurget, 1995). For example, HeLa cells were more sensitive to the cytotoxic effect of chloramphenicol (CAP) after being infected with a CAP-resistant strain of *M. hyorhinis* (Doersen and Stanbridge, 1981). The growth of normal human embryonic lung fibroblast (HAIN-55) cells was inhibited when they were inoculated with large amount of *Mycoplasma hominis* (more than 10^7 cfu/ml) (Sasaki et al., 1981). Arginine deiminase，an enzyme

derived from *Mycoplasma arginini,* strongly inhibited the growth of human T cells and T lymphoblastoid cell lines (Komada et al., 1997). Potential malignant transformation is also an issue in persistent mycoplasma infected cell cultures. Infection of C3H mouse embryonic cells with *M. fermentans* or *M. penetrans*, two mycoplasmas found in unusually high frequencies among patients with AIDS, caused progressive malignant transformation of C3H cells. The transformation was reversible within 6 passages (1 week per passage) of infection. However, the transformation became permanent after 11 passages of infection. The malignant transformation was not caused by integration of mycoplasmal gene(s) into the mammalian cell genome, because no mycoplasmal gene was found in the transformed cells (Tsai et al., 1995; Zhang et al., 1998).

Most mycoplasmas live as commensals. However, infections by pathogenic mycoplasmas can induce host inflammation (Kennedy and Ball, 1987; Lindsey and Cassell, 1973; Peltier et al., 2003; Rollins et al., 1986). Mycoplasma infections are usually chronic and the damage from mycoplasma infections is more likely due to the host immune and inflammatory responses than of the direct toxic effect of mycoplasma (Avron and Gallily, 1995; Razin et al., 1998). It has been shown that murine macrophages and human monocytes produce proinflammatory cytokines and chemokines, including interleukin-1 (IL-1), IL-6, tumor necrosis factor α (TNF-α), and prostaglandins as well as nitric oxide (NO) after being stimulated by mycoplasmas or extracts of mycoplasma membranes (Avron and Gallily, 1995; Gallily et al., 1992; Muhlradt and Schade, 1991; Sher et al., 1990).

The most relevant mycoplasmal components to activate the early host inflammation reaction are mycoplasmal lipoproteins and lipopeptides (Luhrmann et al., 2002). In 1991, a high-molecular-weight material (MDHM), which is a mixture of lipoproteins, was extracted from *Mycoplasma fermentans* (*M. fermentans*). MDHM can induce TNF-α, IL-1 and IL-6 production in cultures of both murine macrophages and human monocytes (Muhlradt and Schade, 1991). Later, the key molecule was identified as macrophage-activating lipopeptide-2 (MALP-2), a truncated lipopeptide representing the N-terminus of MALP-404 lipoprotein (Muhlradt and Frisch, 1994; Muhlradt et al., 1997). Its primary structure was determined as (S-[2,3-bisacryl(C16:0/C18:0;C18:1)-oxypropyl]cystine-GNNDESNISFKEK (Muhlradt et al., 1997). MALP-2 exhibits very high macrophage stimulating activity (MSA), with a

reported half maximal activity at a concentration of 10^{-11}M. That the lipid moiety is very important to the MSA of MALP-2 was demonstrated because nonlipidated MALP-2 was unable to activate macrophages (Muhlradt et al., 1997). MALP-2 is used as a representative molecule by many researchers for studies of mycoplasmal lipoproteins and lipopeptides.

The effects of MALP-2 on host or cultured macrophages are similar to that of bacterial endotoxin lipopolysaccharide (LPS). Both MALP-2 and LPS are macrophage activators and have the ability to induce proinflammatory cytokines and chemokines such as IL-6 and TNF-α (Deiters et al., 2003; Galanos et al., 2000; Yamamoto et al., 2004). Both MALP-2 and LPS signal through the receptor family of Toll-like receptors (TLRs). The difference is that LPS acts through TLR4, while MALP-2 utilizes TLR2/6 (Kraatz et al., 1999; Morr et al., 2002; Poltorak et al., 1998; Takeuchi et al., 2001). Whereas LPS can activate expression of genes through pathways other than the myeloid differentiation factor 88 (MyD88), no MyD88-independent pathway for MALP-2 has been reported (Kawai et al., 2001).

The potential use of MALP-2 for the benefit of human health was reported when MALP-2 was proposed to be used to protect patients from gram-negative septic shock, based on the observation that it induces LPS cross tolerance in mice and has less systemic toxicity as well as pyrogenicity (Deiters et al., 2003). MALP-2 is also used as a potent mucosal adjuvant (Rharbaoui et al., 2002).

1.7 Inflammation

Inflammation is the response of host tissues to challenges by microbial infections or tissue injury. It is a coordinated process that involves the release of exogenous and endogenous chemical mediators, recruitment of leucocytes, and damage and destruction of the host tissues. In extremely serious cases, multiple organ failure or even death can occur (Barton, 2008; Nathan, 2002; Serhan et al., 2008). Knowledge about inflammation dates back to ancient time, in part because it is such a common problem. Celsus documented the tissue response to injury as early as in the 1st century AD (Libby, 2007). The well-established signs of inflammation are: *rubor* (redness, caused by hyperemia), *tumor* (swelling, due to increased permeability and leakage of the microvasculature), *calor* (heat, due to increased blood flow

and the metabolic activity of the inflammatory mediators), *dolor* (pain, in part due to changes in the perivasculature and associated nerve endings), and *functio laesa* (dysfunction of the involved organs) (Libby, 2007; Serhan et al., 2008).

The first step of the inflammatory response to infection is recognition of the infection by the immune system. The innate immune system responds to microbial invasion rapidly by way of its receptors (Libby, 2007; Medzhitov, 2007). The innate immune response receptors target highly conserved features of microbes that are foreign to mammals, called pathogen-associated molecular patterns (PAMPs) (Akira et al., 2001; Medzhitov, 2001). Different from the adaptive immune response receptors, innate immune response receptors lack specificity. Instead they are able to detect a broad range of microbial diversity (Libby, 2007). This strategy is called "pattern recognition" and the receptors are termed "pattern-recognition receptors" (PRRs). An example of a PAMP-PRR pair is LPS from gram-negative bacteria and the receptor TLR4 (Barton, 2008). In certain cases, sterile inflammation can happen when cell death occurs without infection, such as occurs as a result of blunt trauma or ischemia-reperfusion (Barton, 2008). Endogenous ligands released by dead cells (such as heat shock proteins) and innate receptors (such as TLR) are suggested to trigger sterile inflammation (Jiang et al., 2005; Mollen et al., 2006).

The toll receptor was first identified in *Droshophila,* and was shown to be important for flies to defend against fungal infection (Hashimoto et al., 1988; Lemaitre et al., 1996). Later, similar proteins were found in mammalian cells and called Toll-like receptors (TLRs), which are closely related to type I transmembrane proteins functioning as the major PRRs (Takeda and Akira, 2005). TLRs are members of the Toll/IL-1 receptor (TIR) family. All TLRs contain a cytoplasmic TIR domain that can mediate the formation of homo- and hetero-dimers (Akira et al., 2001). Activation of the TLRs is mainly through the IκB kinase (IKK), MAPK and PI3K/Akt pathways (Krishnan et al., 2007). It was also reported that transcription factors NFκB and IFN-regulatory factor (IRF) are activated by the TLRs to regulate the expression of target genes including a large number of cytokine genes (Barton, 2008; Krishnan et al., 2007). Although the TLRs utilize similar signaling pathways, the extracellular structures of TLRs are diverse, which enables individual TLRs to recognize distinct conserved microbial targets (Misch and Hawn, 2008). The well characterized

receptor-ligand pairs are TLR1/TLR2/TLR6 and lipoproteins, TLR3 and double-stranded RNA, TLR4 and LPS, TLR5 and flagellin, TLR7/TLR8 and single-stranded RNA, and TLR9 and CpG motifs in DNA (Kawai and Akira, 2007; Krishnan et al., 2007).

The most influential cells in resolving infections are tissue-resident macrophages and dendritic cells (DCs) that act by signaling through the PRRs on their surface (Mellman and Steinman, 2001). Activation of macrophages and DCs results in the release of proinflammatory cytokines and mediators that profoundly change the local environment of the surrounding tissue and vasculature (Nathan, 2002).

The second step of inflammation is the recruitment of inflammatory cells to the site of infection. The local tissue is in an inflamed state because of a series of morphological and molecular changes of local blood vessel endothelial cells induced by proinflammatory cytokines (such as IL-1, IL-6 and TNFα) and lipid mediators (Barton, 2008). Neutrophils are attracted to the infection site by following the chemotactic gradients (Smith, 1994). Neutrophils are the most abundant leucocytes, representing 50 to 60% of the total circulating leucocytes. They arrive within hours and are the first leucocytes to enter the inflamed site (Schleimer et al., 1989; Smith, 1994). Their primary function is phagocytosis of microbial organisms. Monocytes are also recruited to the inflamed site by the same inflammatory signals but they arrive later than neutrophils and mature into macrophages after they have reached the infection site (Nathan, 2006).. They are also phagocytotic and can phagocytose microbes (Singh et al., 2004).

The third step in resolving infection is the elimination of microbes by the leucocytes, especially the neutrophils and macrophages, that were recruited to the infection site,. Neutrophils fight microbes with their hydrolytic enzymes and antimicrobial polypeptides that are present in intracellular granules, reactive oxygen species (ROS), reactive nitrogen species (RNS) (Smith, 1994). After phagocytosis, neutrophils direct the proteases, ROS and RNS to the phagocytosed microbes to denature their proteins, disrupt lipids and damage their DNA (Nathan and Shiloh, 2000). Neutrophils can also release these active substrates into the extracellular space of the inflamed site. This helps to kill nearby microbes but can also cause damage to the host tissues (Nathan, 2006). Neutrophils will eventually die by apoptosis in the inflamed site. Their debris is then removed by macrophages (Singh et al., 2004).

Macrophages can also phagocytose microbes and kill them by mechanisms similar to those used by neutrophils (Barton, 2008).

The last step in the progression of inflammation is resolution, which is the ideal outcome of acute inflammation (Serhan et al., 2008). Removal of the foreign offenders is critical to resolution, which is a process conducted mainly by neutrophils and macrophages (Barton, 2008; Nathan and Shiloh, 2000). Resolution also requires the removal and reduction of leucocytes and their debris (Henson, 2005). Neutrophil debris is removed by macrophages and living neutrophils can return to the blood. Macrophages use the lymphatic drainage system to leave the inflamed site after finishing phagocytosis, although a small proportion may die locally by apoptosis (Serhan et al., 2007). Another important aspect for resolution is to turn off the proinflammatory signals by anti-inflammatory mediators so as to stop the entry of new leucocytes into the inflamed site (Henson, 2005). For example, lipoxins are anti-inflammatory mediators produced from arachidonic acid by lipoxygenases (Serhan, 2005; Serhan and Savill, 2005). They help to reduce the influx of neutrophils and increase the influx of macrophages to clean the inflamed site (Serhan, 2005). Inhibition of the TLR signaling pathway also helps to achieve resolution. For example, A20, a ubiquitin ligase induced by NFκB, functions as a negative feedback regulator of TLR signaling through degradation of NFκB (Lawrence and Gilroy, 2007).

Complete resolution is achieved and homeostasis is restored if all processes mentioned above are followed. However, acute inflammation has other fates if the injury is too extensive or persistent. Fibrosis, sclerosis or scaring can result from the host's attempts to maintain structural integrity by using fibrotic material for replacement (Henson, 2005). Chronic inflammation can be another fate of acute inflammation if it persists for a prolonged time (Lawrence and Gilroy, 2007).

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CHAPTER 2. PARITY IN THE MAMMARY GLAND IS ASSOCIATED WITH AN EXPANDED MACROPHAGE POPULATION

A paper to be submitted to journal X

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2.1 ABSTRACT

Pregnancy is a well established protective factor against breast cancer. One explanation for this protection is the increased differentiation status of the parous epithelium. However, this does not explain the association of parity with increased aggressiveness of breast cancers, particularly cancers that occur soon after pregnancy. Because tumor aggressiveness can be influenced by the cell population that surrounds the mammary epithelium, we examined the potential role of the immune system in establishing a long-term difference between the mammary glands of parous and virgin animals. Specific mRNA levels, enzyme activities and antigen expressing cells were quantified in parous and virgin mammary glands from Spraque-Dawley rats in diestrous. Our results support the hypothesis that macrophages, but not neutrophils or B-cells, are specifically increased in fully involuted glands compared with age-matched virgin mammary glands. Our finding of an increased macrophage population in the parous breast could explain the dichotomy of reported

association of parity with decreased breast cancer incidence and increased cancer aggressiveness, because of the dual role played by macrophages in opposing and supporting tumor progression.

2.2 INTRODUCTION

It is well established that parity conveys protection against cancers of the breast and other reproductive tissues. This correlation has been demonstrated in many studies using rodent models and from epidemiological analyses of human populations. Full-term pregnancy seems necessary for protection against breast cancer (27, 36). However, the age at first parity affects both the premenopausal and postmenopausal risk for breast cancer (8). A meta-analysis of the compiled data from 47 epidemiological studies in 30 countries, which included 50302 women with breast cancer and 96973 women without breast cancer showed a 4.3% decrease in the relative risk of breast cancer for every 12 months of breastfeeding in addition to a 7.0% decrease in breast cancer risk for each birth (1). The study also showed that protection from cancer obtained by breast feeding or pregnancy was the same in developed and undeveloped countries. The authors concluded that almost 70% of the increased protection against breast cancer observed in parous females of developing countries can be accounted for by the larger number of births per female and the increased frequency of breast feeding by these latter populations (1).

Although parity protects against the occurrence of breast cancer, it is positively associated with increased aggressiveness of cancer and decreased cancer survival rates (2, 9, 20, 22, 45). Thus, the effect of reproductive status on breast cancer is complex with full-term pregnancy and lactation contributing to decreased cancer incidence and increased tumor aggressiveness.

A change in the differentiation state of the mammary epithelium or another cell type has been suggested as the reason for parity-induced protection against tumor incidence (35) and increased p53 expression was proposed as the mediator of this effect (29). However, an

increased differentiation status of the mammary epithelium does not explain the increased incidence of aggressive tumors in parous compared with nulli-parous females.

A microarray analysis of the mouse identified a small group of genes that remain elevated in the fully involuted mouse breast (10). The expression levels of a number of epithelial growth factor genes were lower in primiparous compared with virgin animals and the expression of some genes associated with lymphocytes and macrophages were elevated. The results of this study suggested the interesting possibility that parity results in an increase in the immune cell population in the mammary gland and that the immune cells influence epithelial differentiation status and tumor aggressiveness.

We evaluated the hypothesis that parity results in an increase in one or more hematopoietic cell type in the mammary gland using the diestrous rat as our model. Comparing breast tissue of primiparous and virgin animals we found, in the former, higher expression of genes that are upregulated during the innate immune response. Our results are consistent with the hypothesis that parity results in a specific increase in the size of the mammary macrophage population. The presence of more macrophages in the parous breast is consistent with more effective immune surveillance and decreased cancer incidence in parous compared with nulli-parous females.

M1 macrophages, that protect against cancer, can be converted by tumor cells to M2 macrophages that are also referred to as tumor-associated macrophages (TAMs). Whereas M1 macrophages engulf tumor cells and protect against the development of cancer, M2 macrophages are believed to promote tumor progression by secreting growth factors and proteases, promoting angiogenesis and suppressing adaptive immunity (40). Cancers that do develop in the parous breast might be expected to be more aggressive due to the increased incidence of macrophages that could be converted to the M2 type and that subsequent influence of these macrophages on breast cancer development. Thus, our observation of a specific increase in the macrophage population in parous breasts leads to an overarching hypothesis to explain the effect of parity on breast cancer that includes the influence of the macrophage on epithelial differentiation, immune surveillance and tumor aggressiveness.

2.3 MATERIALS AND METHODS

Materials

Experimental Animals and Materials

Female Sprague-Dawley rats were obtained from Harlan Company (Indianapolis, IN). The rats were allowed to undergo a 21 day lactation period after delivery. Rats were then allowed at least 28 days for complete involution of the mammary gland before being sacrificed. Virgin rats were sacrificed in the same week as the primiparous rats. All rats (primiparous and virgin) were sacrificed on the second day of diestrus, which was confirmed by vaginal smear. Rats were 135 days old when sacrificed.

All animals were housed and treated according to current NIH guidelines. Animal care was provided by an animal caretaker and an attending veterinarian. This research was conducted in accordance with the standards set forth in the NIH guide for the care and use of laboratory animals. Animals were killed by CO_2 inhalation prior to removal of tissues for the described studies. Prior approval was obtained from the appropriate university Committee on Animal Care for all procedures performed on the animals used in these studies.

Unless another source is identified in the text, all chemicals used were reagent grade or higher and purchased from Sigma (St. Louis, MO) or Fisher (Fisher Scientific Inc. Fair Lawn, NJ).

RNA Extraction and Northern Blots

Tissues were frozen immediately in liquid nitrogen upon removal from the animal and stored at -80 ^{o}C. Total RNA was isolated using Trizol Reagent (Invitrogen, Carlsbad, CA) following the manufacturer's instructions. Northern blots were performed as previously described with 15 μg total RNA per lane (37). The blots were hybridized with the following ^{32}P-labeled probes produced by using the Megaprime DNA labeling system (Amersham Pharmacia Biotech, Little Chalfont, Buckinghamshire, England) with the following templates: Lcn2 (cDNA PCR product), SGP2 (EcoR I fragment from SGP-2 plasmid), GAPDH probe (XbaI-PstI fragment of a GAPDH plasmid). Between hybridizations, the membranes were

stripped by boiling 2 consecutive times in 0.1 X SSC with 0.1% SDS for 3 min. Data were obtained by exposure to a phosphorimager (Molecular Dynamics, Sunnyvale, CA).

Real-time RT-PCR

Reverse transcription was done as described previously (12). Briefly, 1μg total RNA was treated with 1 unit DNase (Invitrogen) for 15 min. After de-activation of the DNase, the reverse transcription was done using superscript II (Invitrogen) with 18mer oligo dT and dNTP. The real-time PCR was done in an Opticon (MJ research, Waltham, MA) or a Mini Opticon (Bio-Rad Laboratories, Hercules, CA) with FullVelocity™ QPCR Master Mix (Stratagene, La Jolla, CA). Cyclophilin was used as the reference gene to normalize gene expression in each sample. Primers used in the experiments were: mouse Lcn2, sense: CAGGCCCAGGACTCAACTCAGAA, anti-sense: TCTGGACCGCATTGCCTGC; cyclophilin, sense: CTTTTCGCCGCTTGCTGCA, anti-sense: ACCACCCTGGCACATG-AATCCT; rat Lcn2, sense: CAGGCCCAGGACTCAACTCAGAA, anti-sense: AGCG-GCTTTGTCTTTCTTTCTGGA; cyclophilin: sense: CTTTTCGCCGCTTGCTGCA, anti-sense: ACCACCCTGGCACATGAATCCT; or sense: TGTTCTTCGACATCACGGCTGAT, anti-sense: GGACTTGCCACCAGTGCCATTA; Igf1 sense: GGCACTCTGCTTGCTCA-CCTT, anti-sense: CGGAAGCAACACTCATCCACA; Lbp sense: CTTGGCGTGGTCA-CGAATGTAT, anti-sense: GGAATGCCTGGAACAGGTTCA; SPP1 sense: ATGAGT-CCTTCACTGCCAGCAC, anti-sense: TCATCGGACTCCTGGCTCTTC; Mmp12 sense: GGTCAAGATGGATGAAGCGGTAT, anti-sense: TCGTAATGTCAGCCTCGCCTT; Mpeg1 sense: AGTGATGGATGCCAAGTGTCCTA, anti-sense: TTGGTGGCAACTTGG-CTCAT; TGFβ3 sense: CTGTTGCGGAGAGAGTCCAACTT, anti-sense: GGTCATCTT-CGTTGTCCACTCCT; Emr1 sense: TTTCCTTGCCTGCTTCTTCT, anti-sense: CCTGTC-TCCGTATTCAGCCA; CD14 sense: TACCGACCATGAAGCTTATGCT, anti-sense: GATTTGCTTCCGTGTCCACA; IL-6 sense: ACAGCCACTGCCTTCCCTACT, anti-sense: GAACTCCAGAAGACCAGAGCAGA.

Myeloperoxidase (MPO) Assay

The MPO assay was performed according to the method of Bradley with minor modifications (5). Briefly, tissues were weighed and homogenized in 1ml ice cold 0.5%

HTAB, 50 mM potassium phosphate, pH 6.0. Homogenized samples were lysed by freeze-thaw and clarified by centrifugation. 20 ul samples were assayed with an incubation time of 10 min. The MPO activity was calculated with the formula: [(abs10min-abs0min)/10]/weight of tissue used/0.0113 = MPO/gram of tissue. Where 0.0113 is a constant and the weight of tissue used = (tissue weight/1000)*20.

Immunohistochemistry

A polyclonal antibody recognizing the macrophage marker CD68 was used as the primary antibody (Santa Cruz Biotechnology, Santa Cruz, CA). Paraffin-embedded tissue sections from 6 mammary glands of parous rats and 8 mammary glands of virgin rats were deparaffinized and rehydrated. Antigen retrieval was done by boiling the sections then storing them at 95ºC in 10 mM Tris-EDTA, pH 9.0. Nova Red (Vector Laboratory, Burlingame, CA) was used for visualizing streptavidin–biotin conjugated horseradish peroxidase. Sections were then counterstained with Shandon Hematoxylin (Fisher Scientific, Itasca, IL) to identify nuclei. Sectioning and staining was done by the Veterinary Pathology Services, Iowa State University.

Macrophages were counted from ten randomly selected fields in each section, one section from each tissue sample. In each field the number of macrophages was counted. The counting was done blind with the identity of each sample only uncovered after the data had been collected. The total number of macrophages from the ten fields of each section was used as the data point for that tissue sample for analysis.

Statistics

The standard deviation for quotient of parous and virgin group was calculated based on the standard deviation of each group (18). P values were calculated by t-test.

2.4 RESULTS

Effects of Parity on the Expression of Immune Response genes by the Mammary Gland

A previous microarray comparison of the genes differentially expressed in mammary glands of parous compared with virgin mice identified a set of genes associated with macrophages that were elevated in the parous murine breasts compared with age-matched virgin breasts (10). The tissues pooled for this study were not of a defined stage in estrous and were done mainly with the mouse, which has not been commonly used as a model for parity-induced changes in breast tissue.

We investigated the differences in gene expression between mammary glands of parous and virgin rats in diestrous with an emphasis on genes expressed by macrophages (Table 1). Many of the genes tested in the rat tissues were the same as those identified by the previous microarray of mouse tissues. However, some genes that are selectively expressed by macrophages were also investigated. The results showed that the levels of several mRNAs that are expressed by macrophages were significantly elevated in the primiparous breasts. These mRNAs included CD14 antigen (CD14), interleukin-6 (IL-6), osteopontin (Spp1/Eta-1), macrophage-specific matrix metalloproteinase (MMP)-12, and macrophage expressed gene 1 (Mpeg1) (Fig. 1). We also found higher expression of Lbp in primiparous breast, but we did not find significant differences in the mRNAs levels for IGF-1,TGFβ3 and SGP2, all of which were previously reported to differ between the mouse parous and virgin tissues. Thus, the consistent finding between this study in the rat and the previous microarray study of the mouse mammary gland was that many macrophage markers increase with parity.

Macrophage markers, but not Neutrophil or B-cell markere, are Elevated In Parous Compared With Virgin Mammary Glands.

The population sizes of immune cells (neutrophils, macrophage, B and T cells) increase in the early stages of mammary gland involution (4, 6, 7, 43). Our results from gene expression studies that suggest an increase in the macrophage population in the parous breast might reflect the retention of a certain proportion of all immune cells after involution is

completed. To determine which immune cell types are selectively retained in the parous breast after the gland had completely involuted, we tested for these cell populations using MPO activity (neutrophils), and the gene markers Emr1 (macrophages), B220 (B cells) and OX40 (T cells) (4, 14, 30). The result showed no differences in the neutrophil and B cell markers between primiparous and virgin mammary glands (Fig. 2). The expression of OX40 was not detectable in any sample (data not shown). By contrast the expression of the macrophage marker, Emr1, was higher in the parous compared to the virgin mammary glands ($p<0.01$).

Macrophage are more Numerous in Parous compared with the Virgin Mammary Glands

The observation of elevated levels in parous glands of mRNAs that define macrophages could be explained by an increase in expression of these genes per cell or by an increase in the number of cells in the tissue. To determine if the macrophage population in primiparous glands is higher than in virgin glands we performed immunohistochemistry to detect and quantify macrophages. The results demonstrated that there were more macrophages in the fully involuted mammary glands than in the age-matched virgin glands ($p<0.01$) (Fig. 3).

The parity-induced changes in gene expression are specific for the mammary gland

To examine the possibility that the parity-induced changes in abundance of macrophage markers in the mammary gland reflects a body-wide change in macrophage content, we examined other tissues for similar differences in level of gene expression. For these studies the Lcn2 and Emr1 were chosen as the test genes and normalized to cyclophilin to examine specific changes in gene expression. As seen in Fig. 4, the difference between parous and virgin tissues in expression of Lcn2 and Emr1 was only observed in the mammary gland showing that the effect of parity is specific for this gland.

2.5 DISCUSSION

The participation of inflammatory cells in mammary gland involution has been reported in several studies in which the results of microarray analysis and histochemical staining indicated growth of the hematopoietic cell population, particularly neutrophils and macrophages, in mammary gland during involution (6, 26, 42). However, all these studies were of mouse mammary glands at early stages of involution (up to 4 days of involution).

The results of the previously cited microarray study in which mammary glands were examined after they were fully involuted, showed increase in markers for macrophages, B-lymphocytes and T-lymphocytes in the involuted compared with the virgin glands (10). However, this study was done with a sample derived from pools of mammary glands from parous or virgin mice. The inclusion in either of these pools of an infected mammary gland would increase the level of expression of many acute phase proteins and immune cell markers in the pool. This or a difference in the species studied could be reasons why we did not observe increases in the levels of expression of several acute phase proteins in rat mammary glands that were observed in the mouse microarray study. On the other hand, our result is consistent with a recent microarray study of rat mammary glands in the expression of Lbp, Mmp12 and Spp1 (44).

A consistent finding in our study and the microarray analysis was of significant increases in the levels of expression of a number of markers for macrophages. From these results we reasoned that one possible explanation for the protective effect of parity against cancer is increased immune surveillance in the parous gland. Therefore, we examined the relative levels of markers for hematopoietic cells in involuted and virgin mammary glands. Neutrophil populations in the tissues were determined by the MPO assay (5, 39) and showed no difference between the primiparous and virgin groups. Analysis by real-time RT-PCR of B220, a B-lymphocyte's marker, also demonstrated no difference between the two groups. Although a microarray study found higher levels of immunoglobulin gene expression after involution compared with virgin mice (10, 42) the results of immunohistochemical staining of a B-cell marker showed no obvious changes in the B-cell number even in mammary glands at early stages of involution (42). Taken together, these results suggest that although

the number of B-cells does not increase in the mammary gland after involution is completed, some immunoglobulin genes are still more active than in the virgin gland.

We also tested the levels of several mRNAs, namely IGF-I, Lbp, Mmp12, Spp1, Tgfβ3 and SGP2, in the virgin and parous glands. These genes all showed altered expression between involuted and virgin mammary glands of mouse (10). Among them, Lbp, Mmp12 and Spp1 also showed higher expression in parous mammary glands than in virgin glands of rat in a recent microarray study (44). We found higher expression of Lbp, Mmp12 and Spp1 in the fully involuted mammary gland but we did not find differences in expression levels of IGF-I, Tgfβ3 and SGP2. Several differences between our results and D'Cruz's may be responsible for our lack of agreement. Some possibilities are 1) experimental animals (rat vs. mouse), 2) method (real-time PCR vs. microarray), 3) estrous stage of animals (diestrous vs. not determined), or 4) an infection in one of the glands in the parity pool for the microarray.

We found higher expression of several macrophage markers (Mpeg1, CD14 and Emr1 (F4/80)) in mammary glands of primiparous compared with virgin rats. This result was also observed in the mouse (10). We confirmed the result by immunohistochemical staining of the macrophage marker, CD68. The increased macrophage population in the parous mammary gland may be macrophages that have remained in the gland beyond involution. Although not supported by earlier studies (23, 34) more recent studies have shown that the number of macrophages in the mammary gland increases during involution (3, 21, 42). Thus, it appears that macrophages enter the mammary gland during involution and a subset remains in the gland even after 28 days of involution in the rat when the mammary gland is considered to be fully involuted.

While parity protects against the occurrence of breast cancer, it is positively associated with increased aggressiveness of cancer and decreased survival rates (2, 9, 20, 22, 45). These studies showed a large increase in cancer aggressiveness in breast cancer of parous compared with nulliparous females within about 6 years of parturition. Although most studies did not show a measurable effect of parity on tumor aggressiveness later in life, at least one study found an association of axillary lymph node involvement with parity in women with 4 pregnancies or more and this association was strongest with older women (22).

The association of parity with increased aggressiveness of breast could be explained by the presence of larger numbers of macrophages in the mammary gland after parturition because macrophages are capable of being converted from the protective M1 macrophage to the tumor promoting M2 macrophage. Macrophages remaining in the mammary gland after pregnancy would contribute to creating a different microenvironment in the tissue than is present in the virgin gland. It has been argued that the reason for a higher frequency of more aggressive cancers after pregnancy is that a wound healing and inflammatory microenvironment is established in the mammary gland during involution and that this microenvironment is conduce to the proliferation of tumor cells (38). The macrophage is a component of both wound healing and inflammatory microenvironments and is the one immune cell type that is elevated in parous compared with age-matched virgin mice,

Studies of the effects of hormonal treatments on the susceptibility to breast cancer have lead to proposals that the extent of differentiation of the epithelium in parous glands is greater than in virgin glands (35). This proposal was supported by the finding that p53 is required to observe the effect of parity on carcinogen-induced breast cancer (24, 41). However, p53 was not amongst the genes found to be differentially expressed in the parous compared with the virgin breast (10). Our finding of an increased number of macrophages in the parous gland is compatible with the hypothesis that epithelial differentiation is more complete in the parous gland and that, although p53 is required for differentiation to occur, the driving force is the increased size of the macrophage population. Macrophages are known to be associated with the terminal end buds and to promote differentiation of the mammary epithelium (15, 17).

During mammary gland involution, a local inflammatory response occurs in the absence of infection (28, 42). Lcn2 is an acute phase protein (APP) with high expression in the mammary gland and uterus during involution (37). Its expression in the fully involuted mammary gland of primiparous rats is higher than in virgin rats. Lcn2 is expressed by epithelial and hematopoietic cell types and induces apoptosis in neutrophils but not in macrophages (11). The persistently high expression of Lcn2 in involuted mammary gland may be a reason that more macrophages but not neutrophils remain after involution is complete. We compared the relative expression of macrophage marker, Emr, and Lcn2 in a

variety of tissues of primiparous and virgin animals and found that the expression of Emr1 is higher in parous compared with virgin mammary glands, but not in other reproductive tissues nor in liver. Lcn2 is highly expressed in these tissues on toxic or endotoxin challenge (liver) or during involution (uterus) or throughout the ovarian cycle (ovary). The similar proportional expression patterns of Emr and Lcn2 in parous compared with virgin animals suggests a relationship.

Parity also provides protection against endometrial cancer and ovarian cancer in humans as it does for breast cancer (13, 16, 19, 25, 31-33). We propose that the protection against breast cancer provided by parity may be due to the increased immune surveillance caused by the increased macrophage population in rat mammary gland after parity. However, we did not find more macrophages in rat uterus and ovary after parity. This may imply that increased immune surveillance provided by macrophage is not a universal protective factor against cancer in different organs after parity and may not be the only mechanism for the protection. However, the lack of evidence of protection against endometrial cancer and ovarian cancer provided by parity in rats makes it difficult to relate our results for these tissues to the results of studies of endometrial and ovarian cancers in women.

In summary, we have found that the number of macrophages is increased in the completely involuted primiparous rat mammary glands compared to age-matched virgin glands. The resulting increased immune surveillance in the mammary gland may play a role in the protective effect of parity against breast cancer. The higher frequency of macrophages in the parous gland may also be responsible for the observed positive association of parity and more aggressive breast cancers.

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2.8 FIGURES

Figure 1. Macrophage gene expression is higher in primiparous mammary glands compared with age-matched virgin glands. Total RNA from mammary glands of rats were analyzed by Northern blot (SGP-2) and real-time RT-PCR (others). mRNA of target genes were normalized to reference gene GPDH (Northern blot) or Cyclophilin (real-time PCR) of the same sample. P: primiparous, V: age matched virgin. 14 parous rats and 9 virgin rats were used to obtain the data.

Figure 2. Macrophage-specific elevation of hematopoietic cell markers in parous mammary glands. Representative markers were tested to determine the population densities of immune cells in the mammary glands of primiparous rats and virgin rats. Real-time RT-PCR was used to detect macrophage marker Emr1 and B cell marker B220. The results were normalized to the level of cyclophilin in the same sample. Neutrophils were detected by the MPO assay. The error bars shows the standard error of the mean. * indicates $p<0.05$. Mac: macrophage, Neu: neutrophil, P: primiparous, V: age-matched virgin.

Figure 3. More macrophages found in parous compared with virgin mammary glands. A: immunohistochemical staining with CD68 of a paraffin-embedded tissue section from a rat mammary gland with 1000x magnification. B: macrophages were counted from randomly selected 10 fields with 400x magnification of each section. The total number of macrophages of the ten fields was used for analysis. Error bars are the standard errors of the mean from 6 primiparous samples and 8 virgin samples respectively. $p<0.01$.

Figure 4. Selective increase in macrophage gene expression in mammary glands of the parous rat. Total RNA from liver, uterus, ovary and mammary gland of rats were analyzed by real-time RT-PCR for Emr1 and Lcn2. The values are the Emr1 or Lcn2 mRNA normalized to cyclophilin mRNA of the same sample. P: primiparous, V: age matched virgin. 14 primiparous and 9 virgin rats were used in the experiment. error bars show the standard error of the mean. * indicates $p<0.05$.

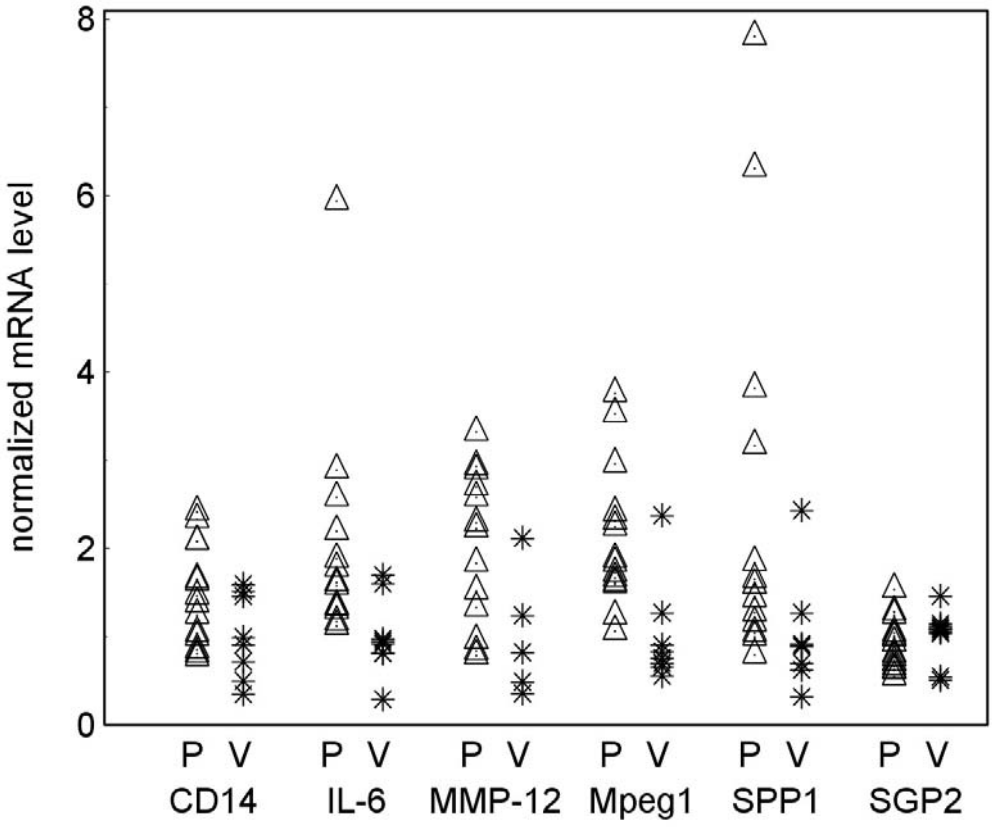

normalized mRNA level
8
6
4
2
0
P V
CD14
P V
IL-6
P V
MMP-12
P V
Mpeg1
P V
SPP1
P V
SGP2

Figure 1

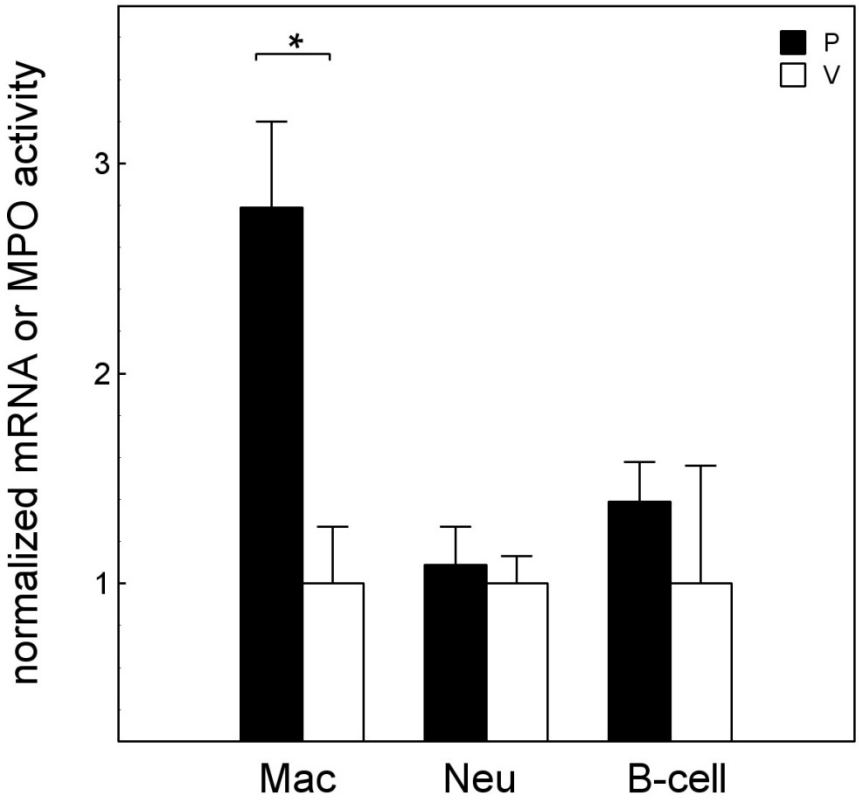


Figure 2

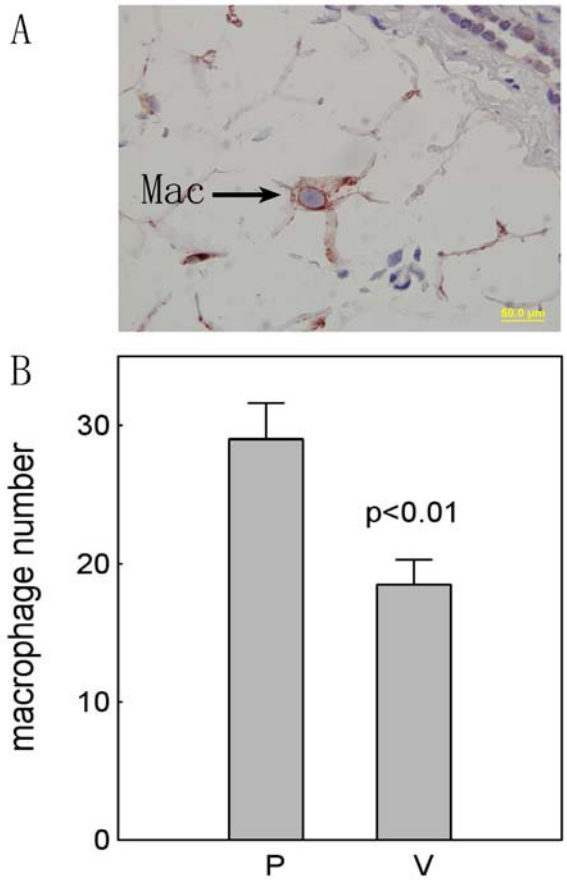
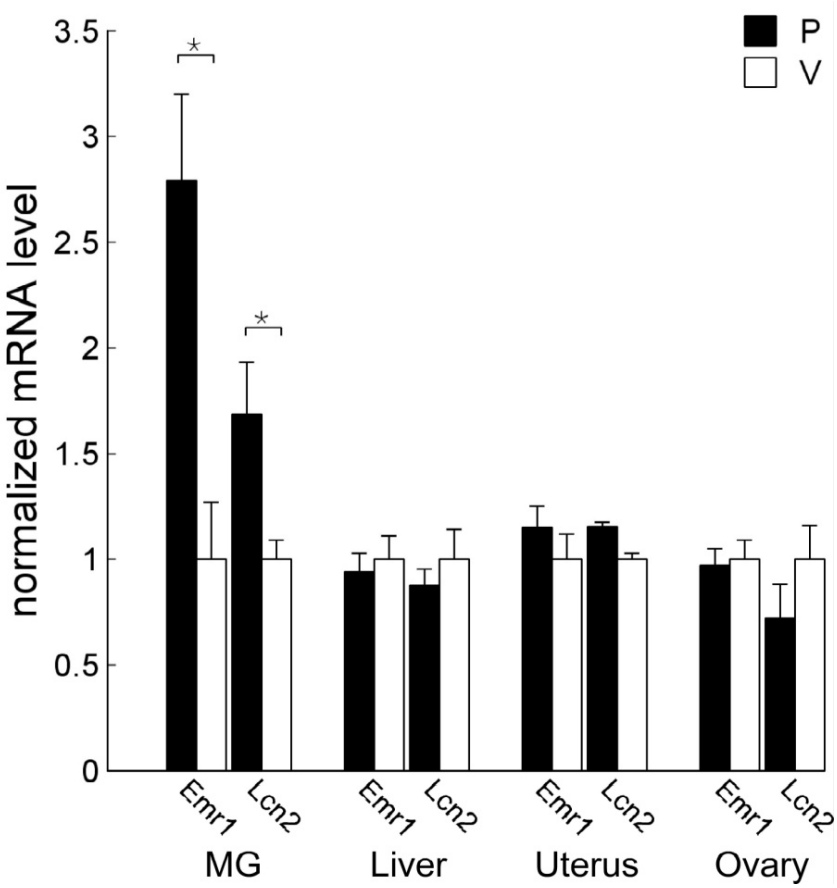


Figure 3


P
V
normalized mRNA level
3.5
3
2.5
2
1.5
1
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*
*
Emr1
Lcn2
Emr1
Lcn2
Emr1
Lcn2
Emr1
Lcn2
MG
Liver
Uterus
Ovary

Figure 4

Table 1. Expression of genes in rat mammary gland after weaning

gene	P/V	std	p value
Emr1	2.79	2.54	0.003*
SPP1	2.57	2.75	0.030*
IL-6	2.07	1.61	0.015*
***Mmp12*	2.05	1.70	0.027*
***Mpeg1*	1.95	1.37	0.010*
Lbp	1.90	1.50	0.017*
Lcn2	1.68	1.05	0.021*
CD14	1.52	0.92	0.042*
SGP-2	0.95	0.41	0.670
IGF-I	0.88	0.41	0.520
Tgfb3	0.52	0.62	0.150

Total RNA from mammary glands of primiparous (P) and age matched virgin (V) rats were analyzed by Northern blot (SGP-2) and real-time RT-PCR (others). mRNA of target genes were normalized to reference gene GPDH (Northern blot) or Cyclophilin (real-time PCR) of the same sample. 14 parous rats and 9 virgin rats were used to obtain the data. Genes expressed by macrophages are in italic. *: $p<0.05$; **: macrophage specific

CHAPTER 3. MYCOPLASMA INFECTION PRODUCES AN INFLAMMATORY STATE OF GENE EXPRESSION IN MAMMARY EPITHELIAL CELLS INVOLVING ACTIVATION BY NFκB, C/EBP AND IκBζ

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3.1 ABSTRACT

Mycoplasma infection causes inflammation of epithelial tissues *in vivo* and is a common problem of cell cultures. Epithelial cells are the first responders to microbial infection when they mount the innate immune response. To understand how mycoplasma infection might influence epithelial cell signaling and gene expression, we investigated the effect of *M. arginini* on mammary epithelial (HC11) cells. Unlike other bacteria, mycoplasmas do not produce the lipopolysaccharides (LPS) that are responsible for activating many signal transduction pathways through TLR receptors. However, macrophage-activating lipopeptide-2 (MALP-2) a mycoplasmal membrane lipopeptide stimulates TLR2/6 and activates downstream genes in immune cells. Here we show that the expression levels of three genes encoding proteins secreted during the innate immune response (Lcn2, IL-6 and TNFα) are elevated in *M. arginini* -infected cells. Expression of these genes was also increased in response to MALP-2. Investigating the mechanism of

MALP-2 action, we demonstrated that MALP-2 induces NFκB, C/EBP and IκBζ. The same changes in gene expression were observed in response to LPS, but with different kinetics. Activation of the Lcn2 promoter requires both NFκB and C/EBP. Selective reduction of IκBζ by shRNA reduced Lcn2 promoter activation by MALP-2. Lcn2 gene expression was persistently activated for at least 72h after adding MALP-2 and was elevated by about 100-fold in cells chronically infected by *M. arginini*. Thus, mycoplasma contamination can significantly change the gene expression pattern of epithelial cells, creating a condition that resembles their activation during the innate immune response.

3.2 INTRODUCTION

Infections by pathogenic mycoplasma can induce inflammation in the host (20, 23, 36, 39). When stimulated by mycoplasmas or extracts of mycoplasma membranes murine macrophages and human monocytes produce proinflammatory cytokines and chemokines, including intenleukin-1 (IL-1), IL-6, tumor necrosis factor (TNFα), and prostaglandins, as well as nitric oxide (NO) (1, 11, 31, 40). The most relevant mycoplasmal components for activating the early host inflammatory reaction are the mycoplasmal lipoproteins and lipopeptides (24). Macrophage activating lipopeptide-2 (MALP-2) is a lipopeptide extracted from *Mycoplasma fermentans* (*M. fermentans*) with the sequence (S-[2,3-bisacyl-(C16:0/C18:0;C18:1)-oxypropyl]cysteine-GNNDESNISFKEK (29, 30). The responses of host or cultured macrophages to MALP-2 are similar to their responses to the bacterial endotoxin, lipopolysaccharide (LPS), and include the production of proinflammatory cytokines and chemokines such as IL-6 and TNFα (7, 10, 43). However, while LPS signals mainly through TLR4, MALP-2 utilizes TLR2 and TLR6 (22, 28, 37, 41).

Epithelial cells *in vivo* are generally the first responders to many pathogenic bacteria. These cells respond with increased levels of the transcription factors NFκB and C/EBP and their regulators such as IκBζ (also called MAIL, "molecule possessing ankyrin-repeats induced by lipopolysaccharide" or INAP, "IL-1-inducible nuclear ankyrin-repeat protein") is induced by pathogens (12, 21, 44). This inflammatory response aids in the resolution of infection.

Mycoplasmas are difficult to detect in cell cultures because they often do not substantially affect the growth rate or cell morphology. Consequently, about 30% of cell lines are contaminated with mycoplasmas (16, 42). Among the more than 100 mycoplasma species, only 5 species, most of human or bovine origin, account for more than 95% of cell culture contaminations (3, 26, 34, 38). Although often not detected in cell cultures, mycoplasma can have profound effects on cells such as increasing their resistance to killing by 5-FUdR and 5-FU (15), degrading extracellular amyloid-beta peptide (45) and inducing differentiation of dendritic cells (5).

HC11 epithelial cells have been used as a cell culture model for differentiated mammary epithelial cells because they have many characteristics that identify them as lactogenic mammary epithelial cells (2, 13). We show here that mycoplasma infection of HC11 epithelial cells in culture induces a response similar to that observed in the innate immune response with activation of the primary response genes, NFκB, C/EBP and IκBζ, and a representative secondary response gene, Lcn2. The results of our studies show how profoundly gene expression can be altered by mycoplasma infection and that the gene expression response in epithelial cells is similar to that in myeloid cells and typical of the innate immune response. We also show that the mycoplasmal product, MALP-2, mediates at least some of its effects on host gene expression by regulating the expression/activity of NFκB, C/EBP and IκBζ in epithelial cells.

3.3 MATERIALS AND METHODS

Materials and Cell Culture

HC11 cells, obtained from Dr. L. Sheffield (University of Wisconsin, Madison, Wisconsin) with permission of the line's originator, Dr. B. Groner (Institute for Experimental Cancer, Freiburg, Germany) (2), were cultured in RPMI 1640 media (Sigma, St. Louis, MO) with 2% FCS (Hyclone, Logan, UT), 5μg/ml insulin (Sigma, St. Louis, MO), 10 units/ml each of penicillin and streptomycin with 5% CO_2. LPS (Sigma) and MALP-2 (Axxora, San Diego, CA) treatments were in the same medium but with 0.1% FCS. HC11 cells were

seeded in 6–well plates cultured for 24 h in 2% FCS culture medium and then switched to 0.1% FCS culture medium. Sixteen hours later, LPS, MALP-2 or vehicle control were added to the medium. Cells were harvested at various times for RNA or protein analysis.

Mycoplasma Detection, Removal and Reinfection

Genomic DNA, prepared using DNAzol (Invitrogen, Carlsbad, CA), was used as template in PCR to detect mycoplasma. The primers in this reaction corresponded to a region of the 16S rDNA that is highly conserved in mycoplasma (4). The primer sequences were: sense ACCATGCACCAYCTGTCAYTC and anti-sense GAGCAAACAGGATTAGATAC. An internal reference control was included with every sample from which a band of 120 bp was amplified. The samples were amplified for 28 cycles of 94 °C for 30 sec, 55 °C for 60 sec, then 72 °C for 60 sec. A band of 280 bp indicates mycoplasma contamination (4). This band, and another region of the mycoplasma 16S rDNA were sequenced and the mycoplasmal contaminant was found to be *M. arginini.*

Cell cultures were cleaned of *M. arginini* by treating the cells with three cycles of 10 μg/ml BM-cyclin 1 (Roche Applied Science, Indianapolis, IN) for 3 days followed by 5 μg/ml BM-cyclin 2 (Roche) for 4 days. The cells were reinfected with *M. arginini* by exposing them to conditioned medium collected from an *M. arginini* infected cell culture that had been filtered through 0.22 μm sterilized filter. The reinfected cells were transferred for three passages before the *M. arginini* could be detected by PCR.

Total RNA Extraction and real-time RT-PCR

Total RNA was extracted by using the Trizol Reagent (Invitrogen) according to the manufacture's instruction. The samples of total RNA were dissolved in DEPC-treated H_2O, quantified by spectrophotometry and stored at -80^{o}C until use. Reverse transcription was done after treating 1 μg total RNA with 1 unit DNase (Invitrogen) for 15 min and inactivating the DNase by exposure to 70 ^{0}C for 15 min. The reverse transcription was done with Superscript II (Invitrogen) with an 18 nt oligo dT as primer. The real-time PCR was done in an Opticon (MJ research, Waltham, MA) using the FullVelocity™ QPCR Master Mix (Stratagene, La Jolla, CA). Cyclophilin was used as the reference gene to normalize gene expression from different samples. Primers used in the experiments were 1) mouse

Lcn2 sense: AATGTCACCTCCATCCTGGTCA, anti-sense: GCGAACTGGTTGTAGTCC-GTGGT; 2) cyclophilin sense: CTTTTCGCCGCTTGCTGCA, anti-sense: ACCACCCTG-GCACATGAATCCT; 3) IL-6 sense: GAGGATACCACTCCCAACAGACC, anti-sense: AAGTGCATCATCGTTGTTCATACA; 4) TNFα sense: CATCTTCTCAA-AATTCGAGTGACAA, anti-sense: TGGGAGTAGACAAGGTACAACCC; 5) IκBζ sense: TGCAGAGGAATCGGCAGTCT, anti-sense: CGGACTGCGTCCAACTGTGT; 6) exogenous IκBζ sense: CACCGCCCTCCATGTTGCT, anti-sense: GCAAACAACAGAT-GGCTGGCA; and 7) eGFP sense: ACAAGCAGAAGAACGGCATC, anti-sense: ACGAACTCCAGCAGGACCAT. For each analysis a standard curve was used to determine the quantity of cDNA in each tested sample. The standard curve was created by amplifying, under the same conditions as the cell-derived samples, a range of known amounts of PCR amplicons (from 10^{-6} to 8 pg/well) using a template with the same sequence as present in the cDNA being quantified. Standard curves were plotted as log(pg/well) vs. C(t). Samples with C(t)'s outside the range of the standard curve were not included in the quantitative data and appropriate dilutions of the cDNA were retested to obtain quantitative values within the range of the standard curve. Triplicate samples were quantified for each RNA preparation and the average of the three values was taken as the value for the RNA preparation.

Luciferase Expression Vectors and Assay

Mouse Lcn2 promoters of various lengths were prepared as PCR products from a mouse genomic BACmid (ES17526, Genomesystems Inc.) template. Fragments were inserted into the luciferase reporter plasmid pGL3-basic (Promega, Madison WI). Primers used for the various truncations were 1) -2108bp, sense: CAGACACAACAGAAGAGGG-CAT, 2) -1477bp, sense: TGTGGGTTGTGTGAGGCTGTA, 3) -1003bp, sense: CAGGGC-AGTGTGGAGACACA, 4) -711bp, sense: GCAGCCACATCTAAGGACTACG, 5) -438bp sense: GGTCTGGTGTTCAGATGGCTT), 6) -253bp sense TGCCTGCCAGAATCCAAAG, 7) -197bp, sense: CAGCCCTTCCTGTTGCTCA, 8) -108bp, sense: GCAATTACTTCATG-GCTTCCTG, 9) -253-NFκB,GGG/AAA: sense: TGCCTGCCAGAATCCAAAGC-CCTAAAAATG with the same anti-sense oligonucleotide starting at +53 bp used for all: GGTTTCCACAGCTACTAGGTCTGA. Mutagenesis of the promoter was done using the

Quickchange II kit (Stratagene) to convert the C/EBP binding site from CAGCCCTTCC-TGTTGCTCAACCTTGCACAGTTCCGAC to CAGCCCTTCCTGGCACTTGGCCTTGC-ACAGTTCCGAC in which the changed bases are underlined.

To measure the relative activities Lcn2 and other promoters, HC11 cells were co-transfected, using Lipofectamine 2000 (Invitrogen), with the appropriate promoter-luciferase reporter plasmids in combination with the plasmid pRLSV-40 (Promega) from which the Renilla luciferase is expressed from the SV40 promoter. The activities of the two luciferases were quantified by the Dual Luciferase assay (Promega) using a Glomax 20/20 luminometer (Promega). The Renilla luciferase activity provides an internal control for cell lysate content of the sample and to obtain a measure of promoter activity. For each sample, the value for the firefly luciferase activity was divided by that for the Renilla luciferase activity. Triplicate samples were quantified for each cell lysate and the average of the three luciferase ratios was taken as the value for the cell lysate.

IκBζ Silent Mutation

IκBζ cDNA was amplified from a preparation of HC11 RNA and was cloned into pcDNA3.1(-) (Invitrogen). A silent mutation in the region that is complementary to shIκBζ in the wildtype mRNA was created using the Quickchange II kit (Stratagene) to convert the sequence GCCCTGCTTCAGAATATTATA to GCCCTGCTCCAAAACATTATA in which the changed bases are underlined.

3.4 RESULTS

Higher Expression of Inflammatory Response Genes in *M. arginini* - Infected Cells

The discovery of a mycoplasma-infected HC11 cell line in our laboratory provided the opportunity of investigating the effect of mycoplasma contamination on epithelial cells and on the expression of genes that are activated during the innate immune response in these cells. The mycoplasma was identified as *M. arginini* by the sequence of its 16S rDNA and

the cells were freed of *M. arginini* with a BM-cyclin regime. A newly infected cell subline was then created using filtered conditioned medium from the parent infected cells. All cell sublines exhibited similar epithelial-type morphology and growth rates (Fig. 1A,B). The infection status of each cell subline was verified by PCR (Fig, 1C)

The levels of expression of three genes that respond to bacterial infection during the innate immune response (Lcn2, TNFα, and IL-6) were determined by real-time RT-PCR. All three genes were expressed at much higher levels in the two cell sublines that were infected with *M. arginini* (Fig. 1D).

Gene Expression Induced by LPS or MALP-2

Mycoplasma do not produce LPS, but they release a lipopeptide, MALP-2, with a similar ability to LPS to induce the expression of IL-6 and TNFα in macrophages (7, 10, 43). Both LPS and MALP-2 increased the expression of IL-6 and TNFα in mycoplasma-free HC11 cells. Whereas the kinetics of the IL-6 responses to MALP-2 and LPS were similar, TNFα gene expression responded to MALP-2 with a peak at least 1 h earlier than for LPS (Fig. 2).

Lcn2, which was more than 100-fold increased in its expression with *M. arginini* infection, was also tested for its response to MALP-2 and LPS. Unlike for IL-6 and TNFα, Lcn2 expression persistently increased over the course of at least 72 h (Fig. 3). This persistent increase in Lcn2 gene expression in response to MALP-2 is consistent with the observed high levels of Lcn2 expression in *M. arginini*-infected cells.

High Sensitivity of Lcn2 Expression to MALP-2

MALP-2 is reported to be a potent macrophage stimulatory lipopeptide that stimulates NO release from macrophages at concentrations in the picomolar range (30). To determine the sensitivity of epithelial cells to MALP-2, we performed a dose response study of MALP-2 activation of Lcn2 expression in mycoplasma-free HC11 cells. The half-maximal response of MALP-2 on Lcn2 expression in HC11 cells was determined from an average of four independent experiments to be 320 pM by using a nonlinear fit model (R^2 0.96) (Fig. 4). These data show that epithelial cells are stimulated by the same concentration

range of MALP-2 that activates macrophages. Thus, *in vivo*, it is expected that both epithelial cells and macrophages are activated by MALP-2 during an *M. arginini* infection.

NFκB and C/EBP DNA Elements are Necessary For *M. arginini* and MALP-2 Induced Lcn2 Expression

To investigate the mechanism by which MALP-2 and *M. arginini* regulate Lcn2 expression we used an Lcn2 promoter driving the expression of a luciferase reporter system. A comparison of reporter activity of a range of truncated Lcn2 promoters showed that the shortest length of promoter to respond to *M. arginini* infection was a 253 bp fragment that contains the NFκB and C/EBP elements (Fig. 5). The NFκB and C/EBP elements in the Lcn2 promoter are important for Lcn2 expression induced by IL-1β and LPS (6, 25).

To test if one or both NFκB and C/EBP elements are required for promoter responsiveness to *M. arginini* infection, reporter plasmids were constructed in which one or both sites were mutated to sequences known not to be bound by the respective transcription factor. These reporter genes were transfected into mycoplasma-free and *M. arginini*-infected cells. The mycoplasma-free cells were also stimulated by MALP-2 to determine the requirement for one or both elements for the MALP-2 response. The results showed that both NFκB and C/EBP elements are required for MALP-2 or *M. arginini* activation of the Lcn2 promoter (Fig. 6).

IκBζ is a MALP-2 Downstream Regulator of Lcn2 Expression

IκBζ is a nuclear member of the IκB family that is reported to cooperate with NFκB in activating gene expression. It has been shown to regulate Lcn2 gene expression in response to LPS in macrophages (19). In Figure 7 it is shown that both MALP-2 and LPS induce IκBζ mRNA which peaked 1 h after addition of MALP-2 and 2 h after addition of LPS (Fig. 7). These time courses parallel those for stimulation of IL-6 and TNFα by MALP-2 and LPS (Fig. 2), suggesting a common mechanism of activation for these three genes.

IκBζ has been shown to be important for regulating secondary response but not primary response genes. Therefore we tested whether IκBζ is involved in the activation by MALP-2 of the secondary response gene, Lcn2. To do this, we used shRNA (short hairpin

RNA) targeted to IκBζ (shIκBζ). To test for the specificity of the shIκBζ in targeting IκBζ mRNA, we created two control plasmids, one that expressed IκBζ mRNA and the other that expressed a mutated IκBζ mRNA in which a silent mutation was introduced into the region complementary to the shIκBζ (mIκBζ). We found that expression of the IκBζ mRNA but not the mIκBζ mRNA was knocked down by shIκBζ (Fig. 8 inset).

The activity of the 253 bp Lcn2 promoter was decreased by 60% when shIκBζ was used to knock down the expression of IκBζ in HC11 cells stimulated by MALP-2 (Fig. 8). Overexpression of either IκBζ or mIκBζ overcame the inhibition of MALP-2-induced Lcn2 promoter activity by shIκBζ. As expected, expression of the mIκBζ, which is not suppressed by shIκBζ, resulted in higher activity of the Lcn2 promoter activity compared to that with IκBζ expression (Fig. 8).

3.5 DISCUSSION

As an externally exposed tissue, the mammary gland is susceptible to bacterial infection, which results in mastitis, an inflammatory condition that has been studied extensively in agricultural animals because of its large economic impact. Infections by pathogenic mycoplasma can also induce host inflammation (20, 23, 36, 39). Tissue-resident macrophages and dendritic cells (DCs) recognize the infection when prokaryotic-derived molecules referred to as PAMPs (pathogen associated molecular patterns) are recognized by pattern recognition receptors such as the Toll-like receptors (TLR). Relevant to this discussion are the mycoplasma-derived MALP-2, which signals through TLR2 and TLR6, and gram negative bacterial-derived LPS, which signals through TLR4 (22, 28, 37, 41). Activation of TLRs results in the release proinflammatory cytokines and other inflammatory mediators (27, 32).

The TLRs are expressed on macrophages, dendritic cells, mast cells and epithelial cells including on mammary epithelial cells (9, 14, 35). Our results show that *M. arginini* infection, MALP-2, and LPS stimulate the expression of genes that are activated by PAMPs and that are induced in epithelial cells during the innate immune response. Although both

MALP-2 and LPS activated the expression of the same three primary response genes (IL-6, TNFα, and IκBζ), the kinetics of changes in TNFα and IκBζ mRNAs were different for LPS and MALP-2 treatment, with the MALP-2 response peaking 1 h earlier than for LPS. This observation suggests that the mechanism leading to increased expression of these two genes differs between LPS and MALP-2. Indeed, it is known that TLR4/6 and TLR2 signaling differs with both utilizing the MyD88-driven activation of NFκB, but only TLR4 signals through IRF3 (33). The kinetics of TLR4 activation by LPS is delayed in macrophages in the absence of MyD88 (18). Therefore, a low level of MyD88 may be responsible for the difference in response kinetics to MALP-2 and LPS in HC11 cells. Although MyD88 has been reported to be expressed in epithelial cells, its expression level in HC11 cells is not known.

In addition to testing the effects of MALP-2 on three primary response genes in HC11 cells (IL-6, TNFα, IκBζ), the ability of MALP-2 to activate a secondary response gene, Lcn2, was also tested. By contrast to the faster response of TNFα and IκBζ, the kinetics of the response of Lcn2 to MALP-2 appeared slower than to LPS. In addition the response to both activators persisted over 72 h with a secondary increase appearing to begin between 48 h and 72 h after addition of the stimulus. This persistent increase was not observed for TNFα and IL-6, both of which are also regulated by C/EBP and NFκB as we have shown here for Lcn2. Instead the persistent increase may be related to the increase in IκBζ, which has recently been shown to activate secondary response genes but not primary response genes in macrophages and macrophage cell lines (19). LPS stimulates a MYD88-dependent trimethylation of H3K4 histone on the Lcn2 promoter, which is proposed to stabilize its activated state (19). By contrast, H3K4 histone methylation was constitutively high on the promoters of two primary response genes. These authors also implicated IκBζ in the mechanism by which LPS activated the Lcn2 promoter by showing that the increase in histone trimethylation on the Lcn2 promoter was lost in bone marrow macrophages from *Nfkbiz*$^{-/-}$ mice (19).

We tested the hypothesis that, as for myeloid cells in response to LPS, IκBζ might also be involved in the MALP-2 regulation of Lcn2 expression in epithelial cells. Two lines of evidence from the studies reported here are consistent with this hypothesis. First MALP-2

induces expression of IκBζ before the maximal increase in Lcn2 is observed. Second, shRNA that selectively decreased the expression of exogenous IκBζ also decreased Lcn2 expression levels. The results of these experiments demonstrate that IκBζ mediates the regulation of Lcn2 by MALP-2 as has been shown previously for LPS. In monocytes, LPS regulates Lcn2 gene expression by histone trimethylation, mediated by MyD88, and through IκBζ by promoting nucleosome remodeling (19). These events of chromosome remodeling may also be downstream of MALP-2 and may be responsible for the persistent activation of this gene in response to this stimulus.

Our results show that *M. arginini* and the mycoplasma membrane lipopeptide MALP-2 induce expression of primary and secondary response genes of innate immunity. The mechanism of induction in mammary epithelial cells is similar to that in myeloid cells in that it requires both NFκB, C/EBP and IκBζ. This result is in contrast to the activation of Lcn2 in Sertoli cells, another epithelial cell type, in which Lcn2 gene expression is regulated by an NFκB-dependent, IκBζ-independent mechanism and is not induced by the inflammatory signals LPS and IL-1β (8).

MALP-2 is active in stimulating Lcn2 at picomolar levels and is thus a very potent inflammatory signal. The dose dependence of the epithelial response to MALP-2 is very similar to the response of macrophages and supports the assumption that the same TLRs are involved in the response in both cell types (30). The persistent activation of Lcn2 in these epithelial cells even after a single treatment with MALP-2 is consistent with the persistent inflammatory response to mycoplasma observed for mammary inflammation due to mycoplasma infections *in vivo* (17).

As well as providing the experimental basis for a mechanistic hypothesis to explain the prolonged activation of Lcn2, and perhaps of other secondary response genes that are activated in epithelial cells during the innate immune response, our results also provide an important reminder that mycoplasma contamination of cell cultures can drastically alter their gene expression profiles without significantly affecting the cell proliferation rate or cell morphology.

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3.8 FIGURES

Figure 1. ***M. arginini* infection does not affect growth rate or morphology of HC11 cells.** HC11 cells infected, cleaned or cleaned and reinfected with *M. arginini* were tested for the levels of expression of key inflammatory response genes. **A.** HC11 cells that were 1) infected with *M. arginini* (myco+), 2) cleaned of *M. arginini* (myco-), or 3) cleaned then reinfected with *M. arginini* (reinfected) were seeded on 10 cm plates. Pictures were taken 48 h after seeding. **B.** HC11 cells were seeded in 24 well plates. Cells were counted after different time points. Shown are the means ±standard deviations (SD) from duplicate wells at each time point from a representative experiment. **C.** Genomic DNAs isolated from the different cell cultures were amplified with primers targeting the 16S rDNA of mycoplasma. PCR products were run in 2% agarose. The mycoplasma DNA was amplified from *M. arginini* infected cell cultures (myco+ and reinfected) but not the mycoplasma-free cell culture. **D.** The expression levels of Lcn2, IL-6 and TNFα and cyclophilin were quantified by real-time RT-PCR. The relative mRNA levels in all samples were normalized to those in cleaned cells. Shown are the mean ± SD from two independent experiments. Myco-: cleaned HC11 cells, myco+: *M. arginini* infected HC11 cells, re-infected: HC11 cells cleaned then reinfected with *M. arginini*.

Figure 2. **IL-6 and TNFα mRNAs are induced by LPS and MALP-2.** Mycoplasma-free HC11 cells were treated with LPS (10 μg/ml) or MALP-2 (10 ng/ml) for the indicated time periods. IL-6, TNFα mRNAs and cyclophilin were quantified by real-time RT-PCR. For each treatment set, the time zero point was used to normalize each set of results at all time points. Shown are the means ± SD from two independent experiments.

Figure 3. Persistent increase in Lcn2 expression induced by LPS and MALP-2. Mycoplasma-free HC11 cells were treated with LPS (10 μg/ml) or MALP-2 (10 ng/ml) for the time periods shown. Lcn2 mRNA was quantified by real-time RT-PCR. Shown are the means ± SD from 2 independent experiments except for the 24 h points, which are the average of 3 experiments ± SD. ○: no treatment, △: LPS, ◆: MALP-2.

Figure 4. Lcn2 gene expression is highly sensitive to MALP-2. Mycoplasma-free HC11 cells were treated with MALP-2 at various concentrations over a 12 h period. Lcn2 and

cyclophilin mRNAs were quantified by real-time RT-PCR. All values were normalized to the average control value from cells treated without MALP-2. Shown are the means ± SD from four independent experiments.

Figure 5. The region of the Lcn2 promoter that responds to *M. arginini* lies between -197 and -253. Cells were co-transfected with a firefly luciferase reporter plasmid containing a truncated Lcn2 promoter (each starting at the listed position and ending at +53 bp) and a Renilla luciferase expression plasmid. The cells were harvested and measured for luciferase activity 48 h after transfection. The relative luciferase activities are shown normalized to the value obtained from cells transfected by a plasmid from which firefly luciferase was expressed from an SV40 promoter. Shown are the means ± SD from four independent experiments for the *M. arginini* contaminated cells (myco+) and two experiments for the mycoplasma-free cells (myco-). Lcn2-1477, Lcn-1003, Lcn2-711 and Lcn2-438 had similar activity levels as Lcn2-2108 in *M. arginini* infected HC11 cells (data not shown).

Figure 6. NFκB and C/EBP binding sites are required for MALP-2-induced Lcn2 expression. Cells were co-transfected with plasmids with the indicated Lcn2 promoters driving firefly luciferase expression and a Renilla luciferase expression plasmid. Twenty four h after transfection the cells were treated with or without 10 ng/ml MALP-2 and then harvested and measured for luciferase activity 12 h later. The relative luciferase activities were normalized to the SV40 promoter control, which was set at 1. Shown are the means ± SD from two experiments. Legend: myco-: mycoplasma-free cells; MALP-2: mycoplasma-free cells treated with MALP-2; myco+: *M. arginini*-infected cells.

Figure 7. IκBζ gene expression is induced by LPS and MALP-2. Mycoplasma-free HC11 cells were treated with 10 μg/ml LPS or 10 ng/ml MALP-2. IκBζ mRNA was quantified by real-time RT-PCR and normalized to the 0-time point. Shown are the means ± SD from two independent experiments.

Figure 8. IκBζ shRNA targets IκBζ mRNA and inhibits Lcn2 promoter activity. HC11 cells were cotransfected with the 253bp Lcn2-luciferase plasmid and Renilla-luciferase plasmid along with combinations of plasmids to express the following RNAs: SHC002 or shIκBζ plus either IκBζ or mIκBζ. pcDNA3.1 was used as a control plasmid for the IκBζ and mIκBζ expression vectors. Cells were treated with 10 ng/ml MALP-2, added 18 h after

transfection, and lysed 24 h later. The lysate was tested for promoter activity by the Dual Luciferase assay. Shown are the means ± SD from two independent experiments. **Inset** shIκBζ selectively targets IκBζ mRNA: HC11 cells were co-transfected with eGFP and a combination of the following: shIκBζ plasmid or a control shRNA plasmid (Sigma), each in combination with the IκBζ or mIκBζ expression plasmids. Samples were collected 24 h after transfection and exogenous IκBζ mRNA was quantified by real-time RT-PCR with plasmid-derived primers that did not amplify the endogenous IκBζ. Each value was normalized to the level of eGFP mRNA in that sample. Shown are the means ± SD from two independent experiments.

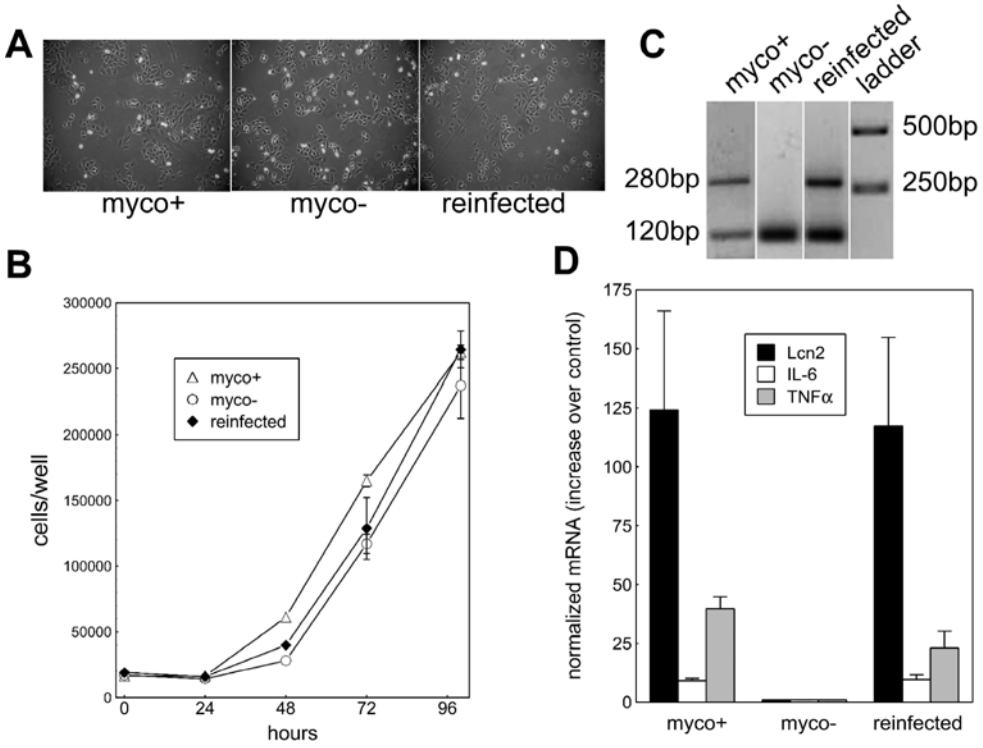
A
myco+
myco-
reinfected
C
myco+
myco-
reinfected
ladder
500bp
250bp
280bp
120bp
B
cells/well
300000
250000
200000
150000
100000
50000
0
myco+
myco-
reinfected
0
24
48
72
96
hours
D
normalized mRNA (increase over control)
175
150
125
100
75
50
25
0
Lcn2
IL-6
TNFα
myco+
myco-
reinfected

Fig. 1

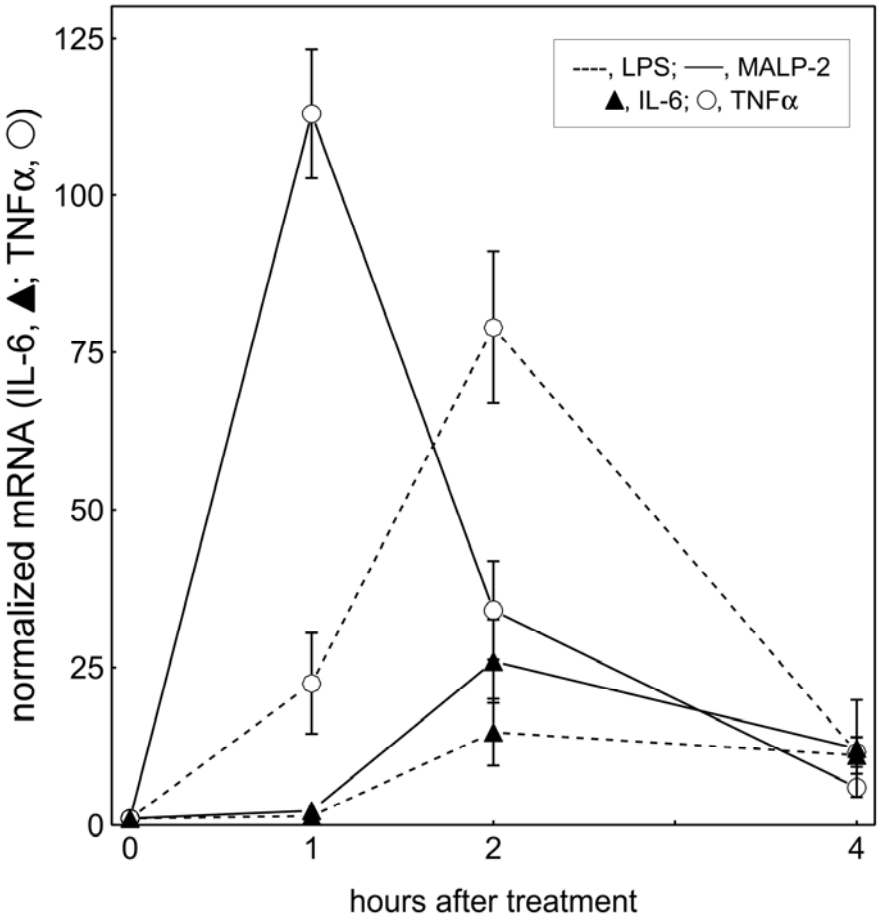
----, LPS; ——, MALP-2
▲, IL-6; ○, TNFα
normalized mRNA (IL-6, ▲; TNFα, ○)
125
100
75
50
25
0
0
1
2
4
hours after treatment

Fig. 2

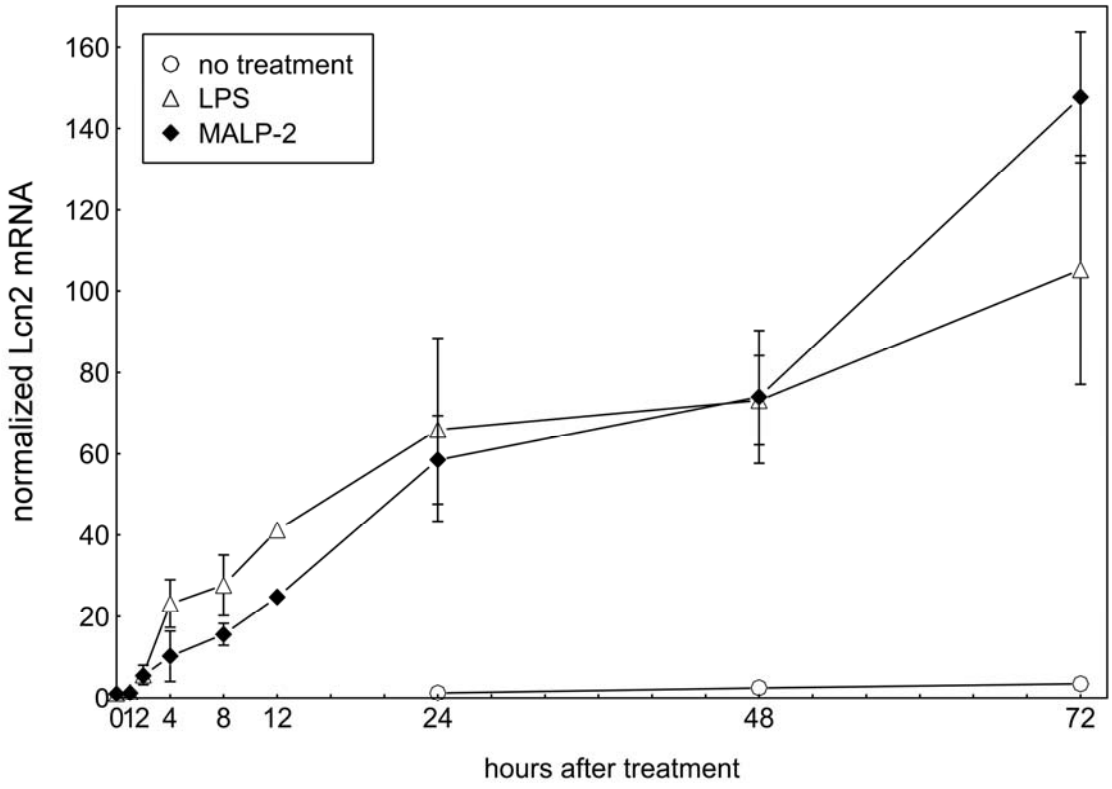
no treatment
LPS
MALP-2
normalized Lcn2 mRNA
0
20
40
60
80
100
120
140
160
0
1
2
4
8
12
24
48
72
hours after treatment

Fig. 3

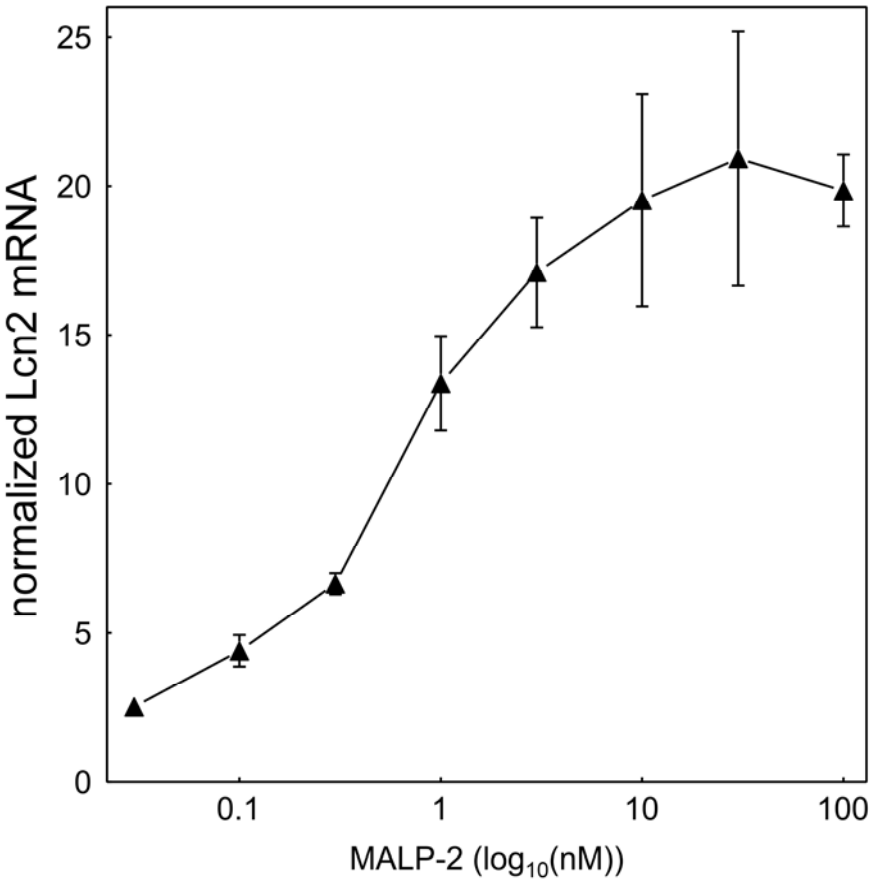

Fig. 4

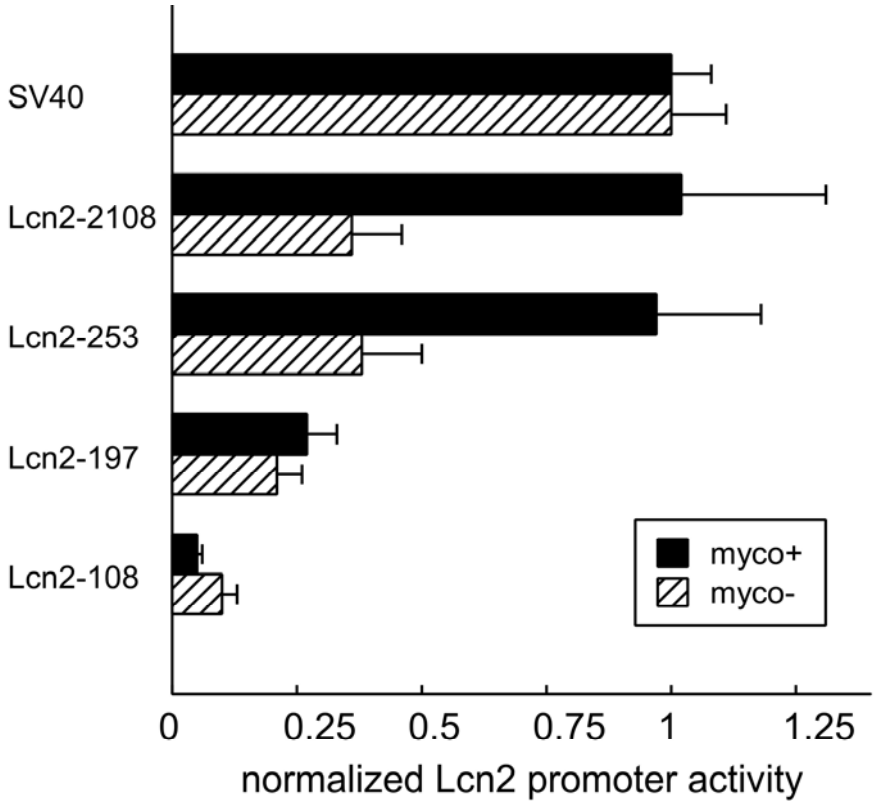
SV40
Lcn2-2108
Lcn2-253
Lcn2-197
Lcn2-108
myco+
myco-
0
0.25
0.5
0.75
1
1.25
normalized Lcn2 promoter activity

Fig. 5

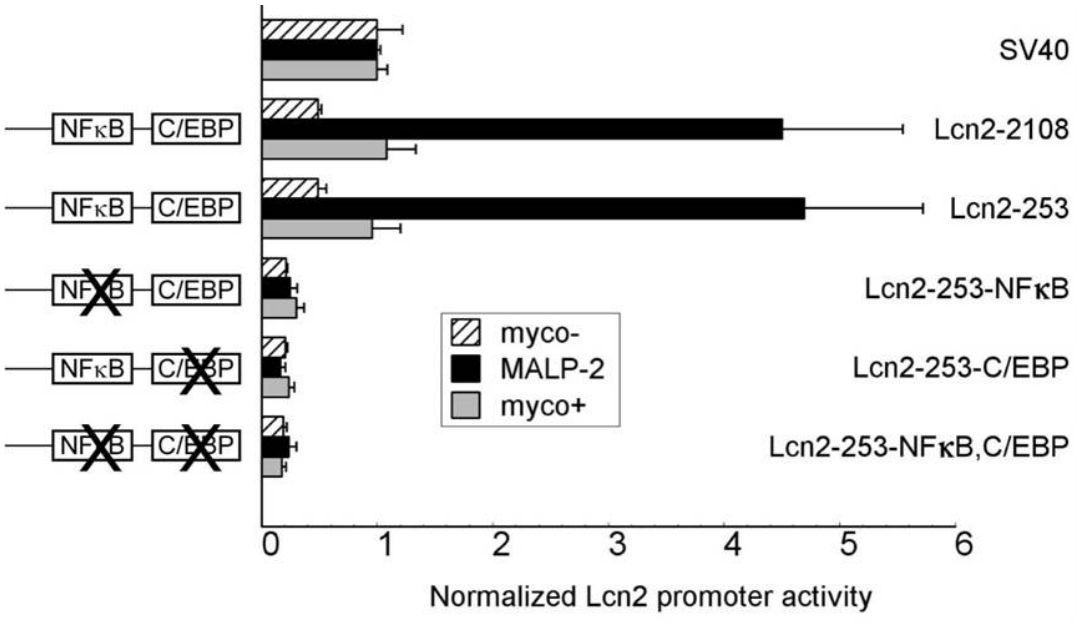
SV40
Lcn2-2108
Lcn2-253
Lcn2-253-NFκB
Lcn2-253-C/EBP
Lcn2-253-NFκB,C/EBP
NFκB
C/EBP
myco-
MALP-2
myco+
0
1
2
3
4
5
6
Normalized Lcn2 promoter activity

Fig. 6

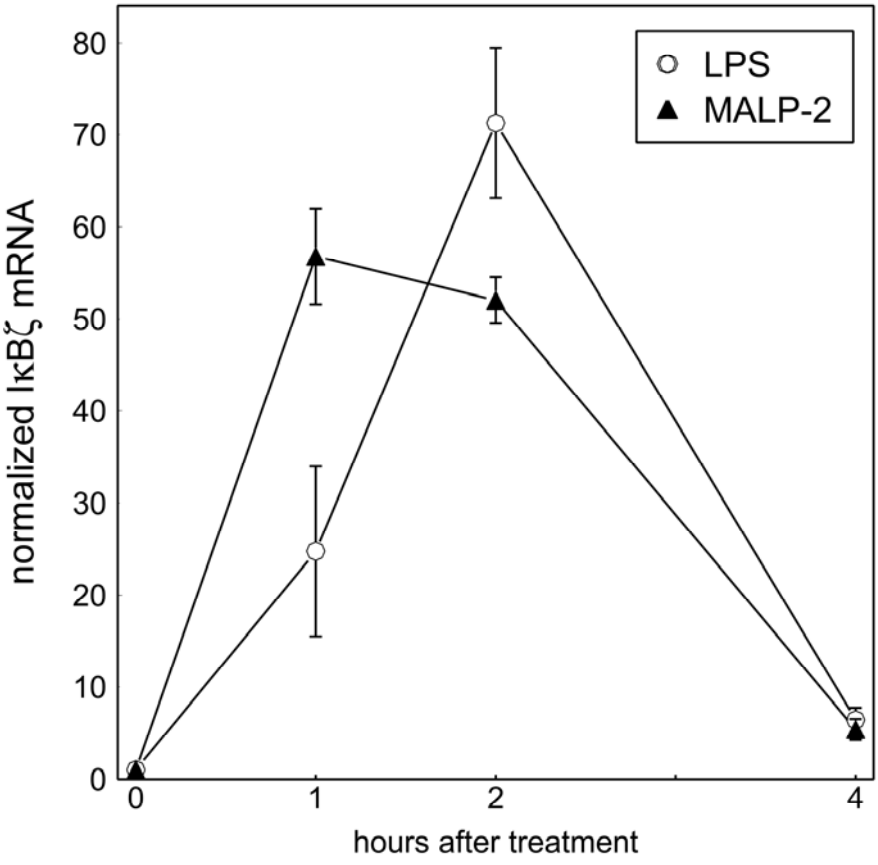
LPS
MALP-2
normalized IκBζ mRNA
hours after treatment
0
10
20
30
40
50
60
70
80
0
1
2
4

Fig. 7

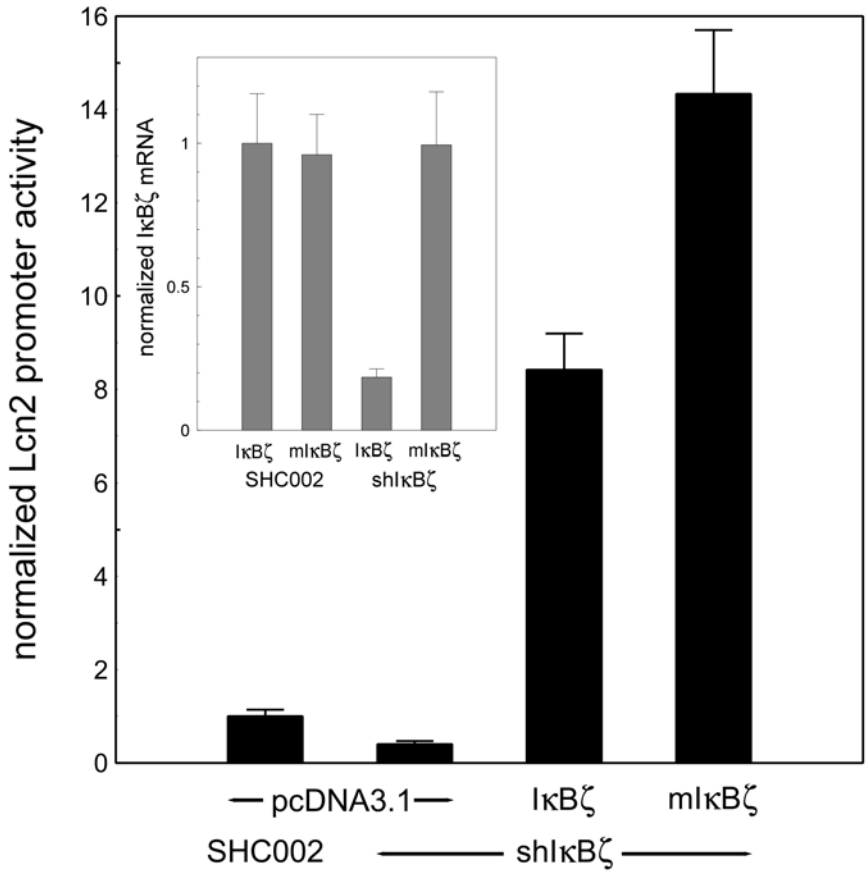
normalized Lcn2 promoter activity
16
14
12
10
8
6
4
2
0
normalized IκBζ mRNA
1
0.5
0
IκBζ
mIκBζ
IκBζ
mIκBζ
SHC002
shIκBζ
pcDNA3.1
IκBζ
mIκBζ
SHC002
shIκBζ

Fig. 8

CHAPTER 4. GENERAL DISCUSSION AND FUTURE PLANS

Elevated Macrophage Population Size and Lcn2 Expression in the Fully Involuted Rat Mammary Gland

One of the strongest protective factors for breast cancer is a full term pregnancy early in life (early 20s) that gives a 50% reduction of lifetime risk of breast cancer when compared with the risk of nulliparous women (Bernstein, 2002; Lambe et al., 1996; MacMahon et al., 1970). The protective effect of an early full-term pregnancy against breast cancer is observed in humans and also in experimental rodent models. Rats and mice that have undergone a full term pregnancy also gain protection against chemically induced mammary carcinogenesis when compared with nulliparous animals (Russo, 1996; Sinha et al., 1988; Welsch, 1985). However, despite the well-documented data, the mechanism of pregnancy/hormone-induced protection against breast cancer still remains unclear. One explanation for this protection is the increased differentiation status of the parous epithelium (Russo and Russo, 1997). However, this does not explain the association of parity with increased aggressiveness of breast cancers, particularly cancers that occur soon after pregnancy (Albrektsen et al., 2006; Colditz and Rosner, 2006; Largent et al., 2005; Manjer et al., 2005; Whiteman et al., 2004).

Studies in the mouse showed that more immune cells infiltrate the mammary gland during involution (Monks et al., 2002; Stein et al., 2004). However, most studies focused on the mammary gland in early stage of involution and only one microarray study indicated that some cells of the immune system may stay in the mammary gland after complete involution (D'Cruz et al., 2002; Monks et al., 2002; Stein et al., 2004). One possible explanation for the protective effect of parity against cancer is increased immune surveillance in the parous gland. Consistent with a previously published finding from the mouse mammary gland (D'Cruz et al., 2002), I also found that the levels of mRNAs from several genes expressed by macrophages were significantly elevated in the primiparous rat mammary gland. I thus tested the hypothesis that parity results in an increase in hematopoietic cell types in the mammary gland using the diestrous rat as the model. The rat is the most commonly used rodent for reproductive studies and for studies of the effect of parity on breast cancer. And to my

knowledge, there is currently no pubulished evidence to demonstrate a change in the immune cell populations in the fully involuted rat mammary gland. My results showed that the number of macrophages, but not B cells, T cells or neutrophils, is higher in fully involuted rat mammary glands compared with age-matched virgin glands.

Lcn2 is an acute phase protein for which expression is up-regulated during early involution (Ryon et al., 2002). I found that Lcn2 expression in fully involuted rat mammary glands is higher than in age matched virgin mammary glands. Lcn2 is expressed by hematopoietic cells responding to infection. However, I did not find an increase of two other acute phase proteins, SGP-2 and Lbp that also respond to infection in parous mammary glands. Thus, the data suggest that the increase in macrophage markers and Lcn2 in the parous mammary gland compared to the virgin gland is not a consequence of infection of the parous mammary glands. The higher expression of Lcn2 in the parous mammary gland may be one reason for the increase of macrophages because Lcn2 has been reported to induce apoptosis in neutrophils but not macrophages.

In summary, I found elevation of both the macrophage population size and Lcn2 expression in the fully involuted rat mammary gland. The increased macrophage population size may provide more effective immune surveillance against arising tumor cells in the parous breast. But, the protective M1-type macrophages can also be converted under the influence of tumor cells to the M2-type that promotes tumor aggressiveness. Thus, an increased macrophage population size in the parous breast could explain the dichotomy of parity association with decreased cancer incidence and increased cancer aggressiveness.

Lcn2 and Inflammation

Inflammation is the response of host tissues to challenges by microbial infections including pathogenic mycoplasma (Kennedy and Ball, 1987; Peltier et al., 2003). Tissue-resident macrophages and dendritic cells (DCs) are believed to mediate recognition of the infection through TLRs on their surface, and release proinflammatory cytokines and other inflammatory mediators (Mellman and Steinman, 2001; Nathan, 2002). After discovering a mycoplasma contaminated cell lines in our lab, I wanted to test whether mammary epithelial cells (HC11 cells) can react to mycoplasma infection. As well as being expressed by the

immune cells, the TLRs are expressed by epithelial cells including mammary epithelial cells (Furrie et al., 2005; Ibeagha-Awemu et al., 2008; Pandey and Agrawal, 2006). I found that HC11 cells have the ability to recognize mycoplasma or MALP-2, a mycoplasmal membrane lipopeptide, and release proinflammatory cytokines such as IL-6 and TNF-α . Expression of Lcn2, an acute phase protein, is also induced by mycoplasma or MALP-2. The role of Lcn2 in the inflammatory response of HC11 cells to mycoplasma or MALP-2 is not clear. However, there are no studies to show mycoplasmas use siderophores to obtain iron. And therefore the role of Lcn2 is unlikely to be to restrict iron availability for mycoplasma as has been demonstrated *E. coli* in mice injected i.p. (Flo et al., 2004). In addition, Lcn2 expression is increased in some sterile inflammatory diseases such as kidney ischemia-reperfusion injury and inflammatory bowel disease (Dooley et al., 2004; Mishra et al., 2006). Thus, Lcn2 may be actively involved in the process of inflammation and its resolution.

I also found NFκB, C/EBP and IκBζ are important for the regulation of Lcn2 expression during the inflammatory response of HC11 cells to mycoplasma infection or MALP-2 treatment. This finding is similar to the regulation of Lcn2 expression by LPS in myeloid cells (Cowland et al., 2006; Matsuo et al., 2007; Sunil et al., 2007; Yamamoto et al., 2004). To our knowledge, this is the first report that IκBζ mediates Lcn2 expression induced by MALP-2. However, the mechanism by which IκBζ mediates Lcn2 expression is unknown. In summary, my results showed that HC11 cells can recognize and produce an inflammatory response to mycoplasma infection or MALP-2.

Future Plans

Pathogenic mycoplasma infection can induce a host inflammatory response, during which the expression levels of primary and secondary response genes increase. The results of this thesis showed that expression of Lcn2, a secondary response gene, induced by MALP-2 is mediated by IκBζ. A recent study showed that nucleosome remodeling and IκBζ-mediated histone H3K4 trimethylation are important for regulation of expression of Lcn2 induced by LPS. I would like to test the hypothesis that nucleosome remodeling and histone H34K trimethylation are also important for expression of Lcn2 induced by MALP-2. This study will help to explain the activation of secondary response genes during mycoplasma infection and

contribute to an understanding of mechanisms of the inflammatory response of the host to mycoplasma infection.

I would also like to investigate the relationship of Lcn2 expression and changes in the populations of hemopoietic cells during mammary gland involution. The population size of both macrophages and neutrophils increases in early involution (Atabai et al., 2007; Clarkson et al., 2004; Clarkson et al., 2006; Stein et al., 2007). But I only found an increase in macrophages in the fully involuted rat mammary gland. I hypothesize that Lcn2 is the reason for there being more macrophages but not neutrophils in the fully involuted rat mammary gland, because Lcn2 only induces neutropil apoptosis. By using the Lcn2 knockout and wild-type mice, we can ask the question of whether Lcn2 expression is a factor that affects the population sizes of hemopoietic cells, especially macrophages, in the mammary gland during and after involution.

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APPENDIX DEVELOPMENT OF LIPOCALIN 2 OVEREXPRESSING MICE

Introduction:

Lipocalin 2 (Lcn2) is a member of the large and diverse lipocalin protein family. Members of this family are usually small extracellular proteins that share a common β-barrel tertiary structure, which forms a binding site for small hydrophobic molecules (Flower et al., 2000; Kjeldsen et al., 1996). Lcn2 is found in human (also named neutrophil gelatinase-associated lipocalin, NGAL), mouse (also named SIP24, 24p3, uterocalin, and siderocalin) and rat (also named neu related lipocalin, NRL) (Goetz et al., 2002; Kjeldsen et al., 1994; Kjeldsen et al., 1993; Nilsen-Hamilton et al., 1982; Stoesz and Gould, 1995). Lcn2 binds iron loaded enterobactin with a K_D 0.41 ± 0.11 nM at 20°C (Goetz et al., 2002). By this means, it is believed to be bacteriostatic because it reduces iron availability to microorganisms. It is thus considered as a component of the innate immune system, which protects the host from invasion of bacteria that use enterobactin to acquire iron (Flo et al., 2004; Goetz et al., 2002). In order to study the function of Lcn2, I generated Lcn2 overexpressing mice.

Materials and Methods

Experimental Animals

All animals were bred and cared for in the Laboratory Animal Facility at Iowa State University and were housed under a 12-h light/dark cycle and treated according to current NIH guidelines. Animal care was provided by an animal caretaker and an attending veterinarian. This research was conducted in accordance with the standards set forth in the NIH guide for the care and use of laboratory animals. Animals were sacrificed by CO_2 inhalation before the removal of tissues for the described studies. Prior approval was obtained from the Iowa State University Committee on Animal Care for all procedures performed on the animals used in these studies.

Lcn2 Overexpressing Mice

Lcn2 overexpressing mice were made by microinjection of a linearized Lcn2 cDNA plasmid fragment driven by the human β-actin promoter and the CMV enhancer into fertilized eggs. The mice were produced by the Mouse Genome Engineering Facillity, U. of Nebraska using DNA constructs provided by me. The genetically engineered mouse strain was backcrossed to the BALB/c strain for 6 generations and the mice were tested for homogeneity by PCR using genomic DNA as template. The primers for testing Lcn2 overexpression were sense AGTCGTCGACGACCTAGTAGCTGTGGAAACCATG and anti-sense CTAAAGCTTTCAGCCACACTCACCACCCA. The transgenic copy number was determined by amplifying with primers of sense TGACAGCAGTCGGTTGGAAGC and anti-sense GGCACGAAGGCTCATCATTCA.

For the transgenic copy number and homozygosity testing, the PCR conditions and cycle numbers were adjusted to make sure that all reactions were still in the exponential phase. Equal volumes of PCR products, standards and samples, were loaded on an agarose gel. Ethidium bromide was used for staining. The picture of the gel was taken and analyzed with Image J software (http://rsb.info.nih.gov/ij/). The primers used to screen homozygotes were the same as used to identify the transgenic animals. The ratio of the transgenic band to the genomic band was compared to that of a known heterozygous mouse. Mice with significantly higher values than the heterozygous mouse standard were identified as possible homozygous mice.

Extraction of Genomic DNA

A 0.5 cm mouse tail fragment was digested by proteinase K (100 mM Tris-HCl pH 8.5, 5 mM EDTA, 0.2 % SDS, 200 mM NaCl, 100 μg/ml proteinase K (Invitrogen, Carlsbad, CA)) at 54°C overnight. Genomic DNA was precipitated with isopropanol, centrifuged at 12000 *x g* at 4°C for 10 min, rinsed with 70% ethanol and centrifuged again. Genomic DNA was then dissolved in 1 mM EDTA, 10 mM Tris-HCl pH 8.0.

RNA Extraction and Quantitative Real-time RT-PCR

Tissues were frozen in liquid nitrogen immediately upon removal from the animals, and then stored at -80^{o}C. Total RNA was isolated with Trizol Reagent (Invitrogen) following the manufacturer's instructions. For reverse transcription, 1 μg total RNA was treated with 1 unit DNase (Invitrogen) for 15 min at room temperature. After deactivation of the DNase with 1 μl 25 mM EDTA and heating at 65 °C for 10 min, the reverse transcription was done by using superscript II (Invitrogen), 18 mer oligo dT and dNTP following the manufacturer's instructions. The quantitative real-time PCR (qrt PCR) was done on an Opticon (MJ research, Waltham, MA) or a Mini Opticon (Bio-Rad Laboratories, Hercules, CA) with the FullVelocity™ QPCR Master Mix (Stratagene, La Jolla, CA). Cyclophilin was used as the reference gene to normalize gene expression from different samples. Primers used in the experiments were: Lcn2, sense CAATGTCACCTCCATCCTGGTCA, anti-sense GCGAACTGGTTGTAGTCCGTGGT; transgenic Lcn2, sense GCCACCACGGACTACA-ACCAGTT, anti-sense CGCTTCCAACCGACTGCTGTCAC; cyclophilin, sense CTTTT-CGCCGCTTGCTGCA, anti-sense ACCACCCTGGCACATGAATCCT.

Results

Development of Lcn2 Overexpressing Mice:

A diagram of the linearized plasmid used to make the Lcn2 overexpressing mice is in Fig. 1A. The transgenic mice were screened by PCR with primers targeting the Lcn2 cDNA insertion. However, we found these primers also gave an amplicon from the wild type mouse genomic DNA. I found there is a linear relationship between this amplification and the amount of genomic DNA used as template. I took advantage of this additional band as an internal control to establish the extent to which the DNA samples were amplified. The F1 mice were of the FVB strain and they were backcrossed to the BALB/c strain for 6 generations before breeding for homozygotes (Fig. 1B).

To determine how many copies of the transgenic Lcn2 gene are inserted into the mouse genomic DNA, the PCR reaction was used to amplify the transgene with primers targeting the β-actin promoter region. Six standards (linearized plasmid diluted in 100 ng

mouse genomic DNA) were used to make a standard curve for calculation. The results showed that the transgenic Lcn2 gene copy number in the transgenic mouse genome is about 20 per haploid genomic DNA (Fig. 1C).

After a homozygous line was established as determined by the PCR test, the homozygosity was confirmed by backcrossing with wild type mice and testing the genotypes of the pups. The result confirmed the mouse line as homozygous (Table 1).

Expression of the Transgenic Lcn2 in Different Tissues

The expression levels of the Lcn2 transgene in various tissues of the transgenic mice were tested. Real-time PCR was used to quantify the amount of Lcn2 mRNA. Primers targeting the 3' UTR of the transgene were used to detect the transgenic Lcn2 mRNA. The results showed that transgenic Lcn2 mRNA was detected in all tissues from transgenic mice but not from wild type mice (Fig. 2A). Heart and lung have the highest transgenic expression level, where the ovary and uterus have the lowest. Other tissues have similar expression levels. To determine whether the transgenic Lcn2 expression alters the total Lcn2 amount, we used primers targeting the Lcn2 coding region to detect both endogenous and transgenic Lcn2 expression. The results showed that the change in Lcn2 expression is different among the tissues. In tissues such as the colon, intestine, heart, kidney, liver and stomach, the total Lcn2 is increased dramatically in tissues from transgenic mouse and Lcn2 was barely detectable in these tissues from wildtype mouse. However, in tissues with high endogenous Lcn2 expression such as the mammary gland, uterus and ovary, there was no difference in total Lcn2 between the transgenic and wildtype mouse (Fig. 2B).

Discussion:

Several Lcn2 functions have been reported including inducing apoptosis in neutrophils, mammary gland epithelial cells and erythroid progenitor cells, preventing apoptosis in human MCF7 (a breast cancer cell line), binding siderophores to reduce iron availability to microorganisms, promoting epithelial healing in the stomach, promoting or inhibiting metastasis of mammary cancer cells (Bong et al., 2004; Devireddy et al., 2001; Fernandez et al., 2005; Goetz et al., 2002; Hanai et al., 2005; Miharada et al., 2005; Playford

et al., 2006; Tong et al., 2005). However, the range of physiologically significant functions of Lcn2 still remains to be defined. For example, the reports regarding the effect of Lcn2 on apoptosis are contradictory and more studies are needed to establish a function (Devireddy et al., 2005; Devireddy et al., 2001; Tong et al., 2003; Tong et al., 2005).

The function of Lcn2 relating to iron binding, transport and as a bacteriostatic agent of the innate immune system is well accepted. However, the proposed endogenous siderophore-like molecule and a signaling Lcn2 receptor has not been identified (Flo et al., 2004; Goetz et al., 2002). In the human, NGAL protects MMP9 and promotes breast cancer metastasis, but the ability to form this covalent bond between NGAL and MMP9 is unique to NGAL and not shared by Lcn2 of other species (Fernandez et al., 2005).

The Lcn2 transgene described here is driven by the human β-actin promoter enhanced by the CMV enhancer. We determined that there are about 20 copies of the transgene inserted into the transgenic mouse haploid genome in a heterozygous transgenic mouse. The transgenic mice were backcrossed to BALB/c strain for 6 generations to have more than 98% of the BALB/c background for our experiments.

The expression of the transgenic Lcn2 gene was tested in ten tissues from homozygous and wild type female mice. Transgenic expression was detected in all tissues tested. In tissues with very low endogenous Lcn2 expression, such as the colon, intestine, heart, kidney, and stomach, the transgenic expression increased Lcn2 dramatically. On the other hand, in tissues with high endogenous Lcn2 expression, such as the mammary gland, uterus and ovary, the transgenic expression did not appreciably affect the total Lcn2 expression level. Therefore these mice will be useful for studies in the colon, intestine, heart, kidney, liver and stomach because the Lcn2 expression in those tissues increased greatly compared to its expression in tissues from the wild type mouse.

It is also interesting to notice the difference of the total Lcn2 expression in different tissues between transgenic and wildtype mice. There are no difference of the total Lcn2 expression between the groups in mammary gland, uterus and ovary, which are tissues with high endogenous Lcn2 expression. Altghou the high endogenous Lcn2 expression may mask the transgenic expression so that we won't see the huge difference between the wild type and transgenic mice, but we should still be able to see difference if the transgenic Lcn2

expression in these tissues is the same level as in heart or lung. However, we did not see the difference. The result also showed that the expression of transgenic Lcn2 is low in uterus, mammary gland and ovary. So the high endogenous Lcn2 expression may exert an inhibitory effect on the transgenic Lcn2 expression, which may be interesting for future study.

Experiments Conducted

I used the Lcn2 overexpressing, knockout and wildtype mice to do two experiments. However, for both experiments more work needs to be done to confirm the results. I thus did not include them in the thesis but give a brief description of the experiments here.

The first experiment was designed to test the hypothesis that Lcn2 expression is a protective factor against breast cancer. Lcn2 knockout and wildtype BALB/c background mice were used in this experiment. They were fed 7,12-dimethylbenz(a)anthracene (DMBA) via gavage. The mice were monitored for breast cancer incidence. However, due to an accidental lapse in the protocol renewal for the experiment, Institutional Animal Care and Use Committee (IACUC) of ISU insisted that the experiment be terminated and all animals sacrificed. The recorded breast cancer incidence was 7 out of total 29 from the Lcn2 knockout mice and 5 out of total 35 from the wildtype mice when I terminated the experiment. However, more wildtype mice than Lcn2 knocked mice were terminated prematurely due to staggered start times. I also found stomach tumors in 6 out of 29 knockout mice and in 0 out of 35 wildtype mice by palpation. Subsequent pathological study confirmed that they were stomach squamous cell carcinoma. Although the wildtype mice may also have had stomach cancers, the tumors were not large enough for me to detect by palpation whereas they could be detected in the knockout mice. This finding should be further explored with a better designed protocol focusing on stomach cancer.

The second experiment was designed to test the hypothesis that Lcn2 is a protective factor against Salmonella infection because Lcn2 has been shown by others to protect mice against infection of E. coli (Flo et al., 2004). *E. coli* utilizes enterobactin to obtain iron and Salmonella also secrets enterobactin. Therefore, the mouse strains were expected to respond similarly to a Salmonella oral challenge as they had been reported to respond to an *E. coli* i.p

challenge. The result showed that 2 out of 5 Lcn2 overexpressing mice, 3 out of 5 wildtype mice and 0 out of 5 Lcn2 knockout mice died by the 6th day after the Salmonella challenge. The result is contradictory to the hypothesis and the reported protection of Lcn2 against infection of *E. coli*. The experiment needs to be repeated with more mice to confirm the finding.

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Figures

Figure 1. Development of Lcn2 transgenic mice. A: Linearized plasmid. Lcn2 cDNA was inserted in the plasmid. Expression was regulated by the CMV enhancer and the human beta actin promoter. The picture is from software vector NTI. B: Screening Lcn2 transgenic mouse. The primers were designed to amplify the inserted Lcn2 cDNA. The 500 bp band is the amplicon from mouse genome DNA and the 663 bp band is from transgenic DNA. wt: wild type; tr: transgenic; B: blank. C: The transgenic Lcn2 copy number determination. A PCR reaction with primers targeted to the transgenic gene promoter region was used to determine how many copies of the transgene were inserted into the mouse genome. Six standards, which were the linearized plasmid diluted to various extents in 100ng mouse genomic DNA, were used. One hundred nanograms of genomic DNAs from heterozygous transgenic mice 4710.27 and 4710.28 were tested. Equal amounts of the PCR products were loaded on 1.2% agarose gel. The picture of the gel was analyzed by Image J for quantification.

Figure 2. Lcn2 expression in transgenic and wild type mice. Lcn2 mRNA was detected by real-time PCR and normalized to cyclophilin for each sample. A: transgenic Lcn2 mRNA from various tissues. B: total Lcn2 mRNA from various tissues. wt: wild type; tr: homozygous transgenic. MG: mammary gland.

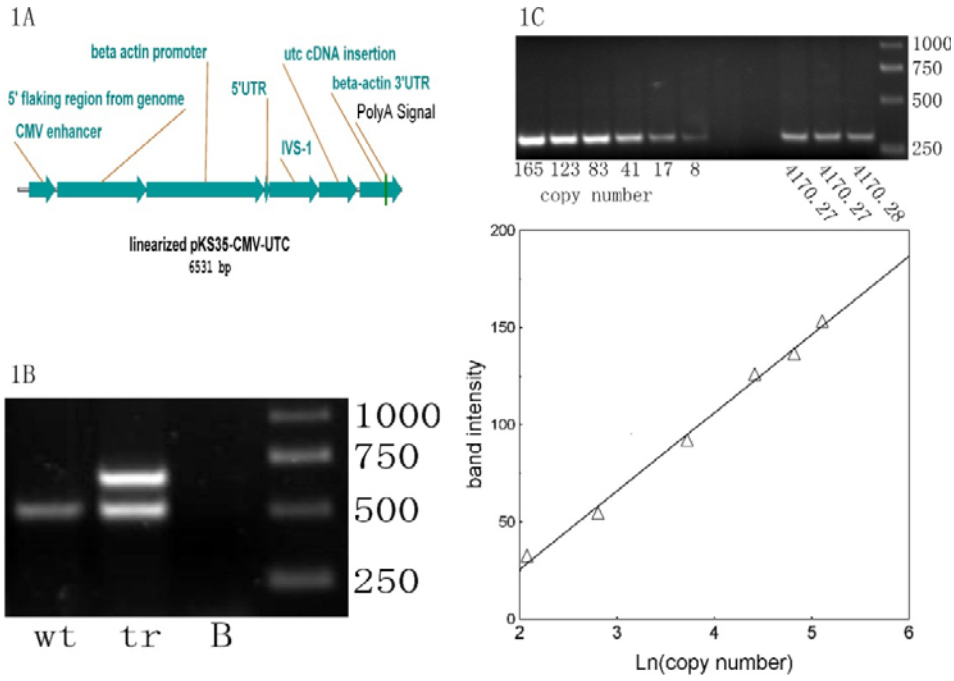
1A
beta actin promoter
utc cDNA insertion
5' flaking region from genome
5'UTR
beta-actin 3'UTR
PolyA Signal
CMV enhancer
IVS-1
linearized pKS35-CMV-UTC
6531 bp
1B
1000
750
500
250
wt tr B
1C
1000
750
500
250
165 123 83 41 17 8
copy number
4170.27
4170.27
4170.28
band intensity
Ln(copy number)

Fig. 1

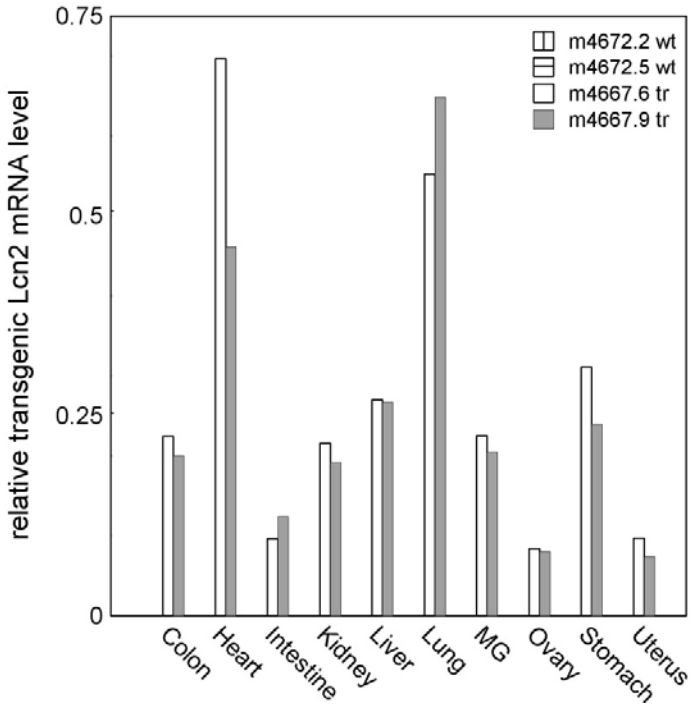
0.75
0.5
0.25
0
relative transgenic Lcn2 mRNA level
m4672.2 wt
m4672.5 wt
m4667.6 tr
m4667.9 tr
Colon
Heart
Intestine
Kidney
Liver
Lung
MG
Ovary
Stomach
Uterus

Fig. 2A

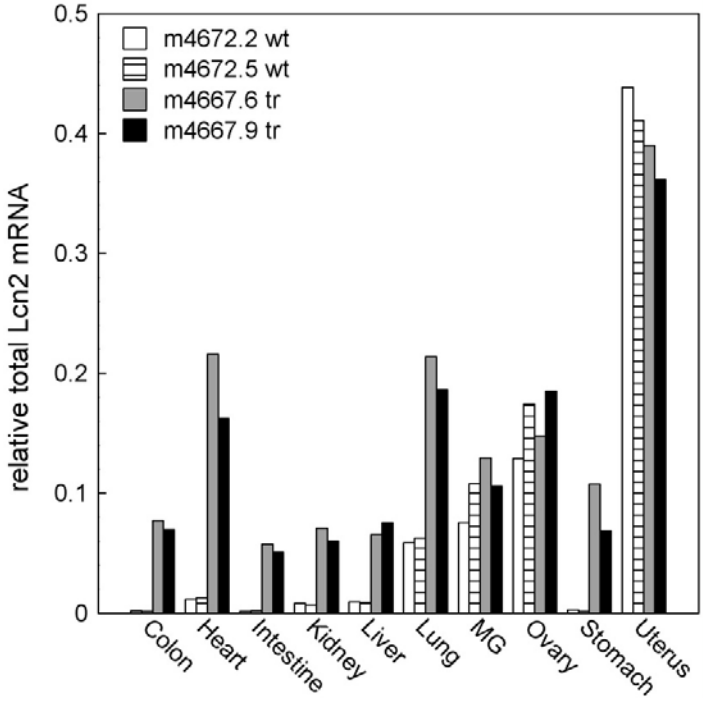
0.5
0.4
0.3
0.2
0.1
0
relative total Lcn2 mRNA
m4672.2 wt
m4672.5 wt
m4667.6 tr
m4667.9 tr
Colon
Heart
Intestine
Kidney
Liver
Lung
MG
Ovary
Stomach
Uterus

Fig. 2B

Table 1 Genetic test for homozygosity

sex	number of pups
F	6
M	8
M	12
F	5
M	11

PCR identified homozygous transgenic mice were mated with wild type mice. The pups were tested for the transgene. All pups were transgenic.

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